DEVELOPMENT AND EVALUATION OF CANDIDATE MICROBIAL SOURCE TRACKING MARKERS TO USE FOLLOWING LAND APPLICATION OF BIOSOLIDS

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A dissertation submitted to the faculty at the University of North Carolina at Chapel Hill in partial fulfillment of the requirements for the degree of Doctor of Philosophy in the Department of Environmental Sciences and Engineering in the Gillings School of Global Public Health.

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ABSTRACT

Patsy M. Polston: Development and Evaluation of Candidate Microbial Source Tracking Markers to Use Following Land Application of Biosolids
(Under the direction of Jill R. Stewart)

Biosolids are generated from the treatment of human waste and upon proper treatment specified by regulatory agencies they are frequently land applied on fields for waste disposal and as a soil amendment. Current methods for assessing water quality around land application sites cannot distinguish biosolid runoff from other sources of pollution. The goal of this research was to *identify, develop, and validate novel biosolid microbial source tracking (MST) markers that can be used for tracking biosolid materials following land application.* Biosolid samples were collected from two different wastewater treatment plants (WWTPs) in the Southeastern region of the US. Total community DNA was extracted and high-throughput 454 pyrosequencing was performed to examine the microbial communities (*Archaea* and Bacteria) present in the samples. Microbial markers were designed and validated using polymerase chain reaction (PCR) for presence/absence in additional biosolids and animal manure samples. Using MST techniques in a field study, surface waters near biosolid land application fields were tested to demonstrate environmental detection of candidate microbial biosolid markers. The combined pyrosequencing and PCR analysis identified several candidate sequences within the *Archaea* and Bacteria kingdoms as potential microbial markers, and upon further *in silico* analysis, we selected sequences belonging to the following genera to target: unclassified

Betaproteobacteria, Leptotrichiaceae, Methanosaeta, and an unclassified, uncultured *Archaea.* The validation study confirmed these microbial biosolid markers are sensitive to biosolid materials. However, initial tests with treated animal wastes suggest that these markers are not specific for biosolid materials but can be found in other digested wastes. The field study resulted in the environmental detection of candidate biosolid markers in surface water samples, along with fecal indicators and other microbes of public health concern. Although sample numbers were small and marker detection was not specific to just biosolids application sites, this research provides an approach for understanding the potential transport of biosolid materials following land application, and potential impacts on environmental quality.

To my mom and in loving memory of my Granny
Because of my grandmother, my mother, and other women's shoulders on which I stand…

I CAN!

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Still I rise…PhD (Perseverance help Determination)!!

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LIST OF ABBREVIATIONS

40 CFR Part 503	Chapter 40 Part 503 of the Code of Federal Regulations
BLAST	Basic Local Alignment Search Tool
CFU	Colony-forming unit
FC	Fecal coliform
LA	Land application
MPN	Most probable number
MST	Microbial source tracking
NCBI	National Center for Biotechnology Information
NRC	National Research Council
OTUs	Operational taxonomic units
PCR	Polymerase chain reaction
PFU	Plaque-forming unit
qPCR	Quantitative polymerase chain reaction
STV	Statistical threshold value
TC	Total coliform
USEPA	United States Environmental Protection Agency
USGS	United States Geological Survey
WWTPs	Wastewater treatment plants

CHAPTER 1. INTRODUCTION

Biosolids are generated during treatment of human sewage. Biosolids may be recycled and used as fertilizer following treatment to reduce the public's risk of environmental exposure to infectious pathogens. The two classes of biosolids, currently classified based on pathogen destruction, are Class A and Class B. Class A biosolids are more highly treated and used without any restrictions at a land application site. They can also be sold to the public through composting companies for application to gardens and yards as a soil amendment. Class B biosolids are treated to reduce pathogens but still contain detectable levels and are, therefore, restricted from use on fields for food crops, for grazing animals, and where there is public contact.

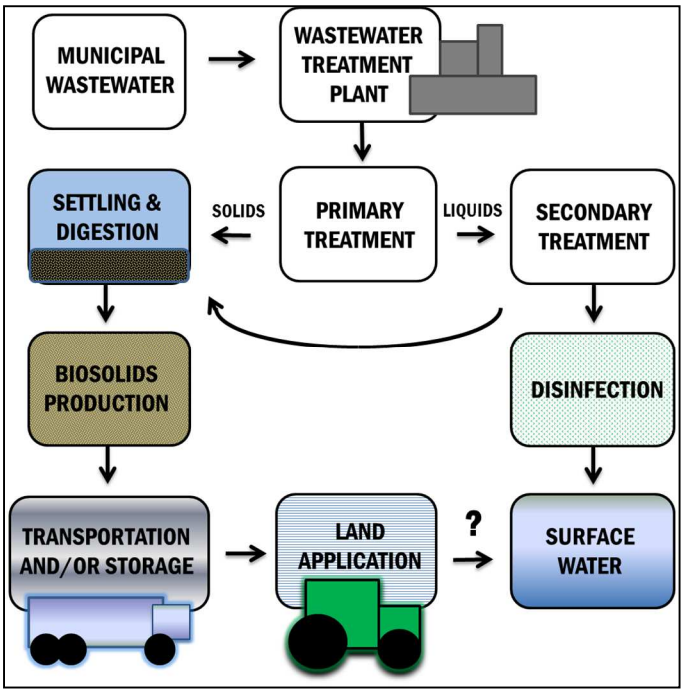

Figure 1. Fate of municipal wastewater and biosolids.

Land Application of Biosolids

Upon proper biosolids treatment, Class A and Class B biosolids are land-applied on fields, adhering to regulations and guidelines established by the USEPA. These regulations and guidelines are encoded in Chapter 40 Part 503 of the Code of

Federal Regulations (40 CFR Part 503).[1] To be classified as Class A, the fecal coliform density must be less than 1000 most probable number (MPN) or *Salmonella* density of less than 3 MPN/4g total solids with no detectable enteric viruses, i.e., less than 1 Plaque Forming Unit (PFU)/4g.[1] For Class B, the density of fecal coliforms must be less than 2,000,000 MPN per gram of total solids.[1] Class B biosolids are most commonly produced in the United States and, therefore, most commonly disposed of by land application.[2]

Land application is the primary means of managing and disposing of biosolids. In the United States approximately 60% of the 5.6 million dry tons of sewage sludge disposed of annually is land-applied.[2,3] However, this practice sometimes generates concern among nearby residents due to potential pollutants associated with biosolids, the lack of evidence about the transport of pollutants, and potential impact on the environment and human health, including quality of life.

Research indicates that fecal pathogens are present in human waste and the application of biosolids may expose humans in the surrounding communities to illnesses through direct ingestion of contaminated surface or groundwater and aerosol exposure. [3] Consequently, many communities near sites where biosolids are being applied are concerned not only about the health impact and the risks associated with biosolids but about other non-pathogenic pollutants (e.g., endotoxins, allergens, irritants, volatile organic carbons, and flame retardants, to name a few). To investigate this risk of exposure, it is important to identify markers that can be used to trace a contaminant to its original source, distinguishing biosolid runoff from other sources of pollution. Additionally, biosolid microbial source tracking

(MST) markers could be used to assess human exposure to non-pathogenic biosolid-associated pollutants.

Fate and Transport of Contaminants Following Land Application

The fate and transport of contaminants in biosolids following land application is not well understood. For example, a recent review noted that a better assessment of specific pathogens and their transport, regrowth, and survival during land application is needed to ensure the safety of our water and food supplies. [4] One possible pathway is transport to surface water following a rain event. Many variables play a role in the ability of contaminants contained in biosolids to migrate to surface waters. These include but are not limited to the dilution factors in waterways, rain intensity, the gradient of the land surface and type of soil to which the biosolids are applied, degradation of the contaminants, and the use of management strategies such as buffer zones.

Traditional Fecal Indicators

Traditional fecal indicators have been used as measures to monitor water systems (drinking, wastewater, and recreational). The majority of biosolids research has been limited to investigating transport of some of these microorganisms following land application. The most frequently researched indicators include total coliforms (TC), which are indicators of fecal contamination but can also be found naturally in the environment; fecal coliforms (FC), *Escherichia coli, and* enterococci*,* which are found in animal and human intestines and feces; and coliphages, which are viruses that infect the bacterium *E. coli* and indicate fecal contamination.[14] Additionally, fecal streptococci, sulphite-reducing clostridia, *Clostridium perfringens*,

bifidobacteria, bacteriophages, and *Bacteroides fragilis* have been used as indicators of fecal contamination.

It is not feasible to test for every indicator, and the majority of biosolids research has been limited to investigating transport of traditional microbial indicators (e.g., fecal coliforms, total coliforms, *E. coli,* enterococci) following land application of biosolids. Traditional indicators are problematic because they are not specific to biosolid materials. Water samples can test positive for fecal contamination because of biosolids or due to other sources of fecal contamination including leaky septic tanks, grazing animals, and birds.[5] It is also suggested that traditional indicators might not survive anaerobic digestion (a typical treatment process for sludge at wastewater treatment plants), aerosolization, and other environmental stressors.[6-8] These factors discourage the continued use of only traditional indicators for investigating transport following land application of biosolids, and some studies have demonstrated the utility of non-traditional microorganisms to detect contaminants following land application. It should be noted that it is not transport of indicators per se that is of concern but the transport of pathogens themselves and/or other contaminants associated with biosolids.

Non-Traditional Microbial Indicators

For the purpose of this research, we focused our attention on microbial indicators and did not choose to research the utility of tracing chemicals (i.e., caffeine) or toxicants associated with human wastes. Also, this is not the first study to try to track microorganisms in the environment following land application. Studies conducted at one of the largest land application sites in the United States detected

hydrogen sulfide–producing bacteria and clostridia and suggested clostridia as a better, more accurate indicator of biosolid contamination.[6-8] This study used a culture-based method for detection and then targeted the 16S-23S-interspacer region for DNA fingerprinting, successfully tracing clostridia to its original biosolids pile. [6-8] In a separate study that was successful in detecting bioaerosols during high wind events following land application, the investigators developed and validated three sensitive and specific biosolid microorganisms: *Clostridium bifermentans, Chloroflexi,* and *Euryarchaeota.*[9] These indicators were unique to biosolids and therefore were good indicators for airborne biosolid contamination. Additionally, this study showed the utility in using a non-culture dependent method for identifying uncultured microorganisms versus traditional indicators that are non-specific and not always present when assayed.[9]

Promising targets for microbial source tracking for human waste involve detection of anaerobic intestinal bacteria because they appear to be host-specific. One of the most promising approaches proposed to track domestic wastewater pollution is detection of the n*if*H gene of *Methanobrevibacter smithii*. [10] *M. smithii* is the most abundant methanogen in the human gut, occurring in concentrations of up to 10^{11} per gram (dry weight). [11] Validation tests of this marker suggest that it is highly specific to human fecal material. [10, 12] Similar research has also identified another sewage marker targeting human-specific genomic sequences from the anaerobic bacteria *Bacteriodales,* specifically for the Bacteriodales marker HF183.[13,14] In a recent methods comparison study with participation of 27 laboratories, the HF183 marker tested as the most sensitive and specific for

identifying human fecal contamination.[14] There are likely other dominant strains of microbes present and potentially other pollutants in treated biosolids that could be used as tracers in the environment. However, to the best of our knowledge, no study has ever evaluated the use of MST techniques to track occurrence of biosolids in surface waters following land application.

Microbial Source Tracking

Tools to track the potential off-site migration of biosolid materials following land application would be transformative in assessing the environmental quality associated with disposal of biosolids. It is not practical to target all pollutants that may be present in biosolid materials. A more viable approach would be to identify a subset of microbial indicators of biosolids that persist as they travel overland and enter nearby surface waters or otherwise might be transported to receptors beyond the application site. MST methods are used to detect the nucleic acids of a microorganism and not the actual organism. The genetic material aids in developing the microbial marker, allowing the organism to be tracked in the environment to its original source, but it does not provide any information pertaining to infectivity. Therefore, potential health risks associated with biosolids' land application cannot be directly measured. Despite not being able to directly detect pathogens or to infer infectivity, MST could provide evidence indicative of the presence of biosolids, which would be helpful for conducting exposure assessments for nearby communities.

Pyrosequencing and Quantitative Polymerase Chain Reaction (qPCR)

Polymerase chain reaction (PCR) and quantitative PCR (qPCR) are molecular detection methods used to identify and quantify microorganisms by

amplifying a single copy of the target DNA to millions of copies of DNA that can be easily quantified. For environmental samples, PCR can be used to determine presence/absence of pathogens, especially viruses or other microorganisms that typically need to be cultured. However, these molecular methods can only amplify specified sequences of nucleic acids. More advanced methods are needed to explore microbial diversity and to understand microbial communities.[15,16]

Pyrosequencing is a robust technique that allows for deep sequencing of the microbial communities, which is needed to characterize microbial diversity.[5,17,18] The 16S rRNA gene, found in almost all bacteria, is often targeted for sequencing studies to investigate similarities or differences in the microbial communities between samples, which leads to the identification of unique and dominant taxonomic groups.[17] Previous studies have demonstrated the effectiveness of pyrosequencing when determining dominant, abundant, and unique members of microbial communities of wastewater influent, activated sludge, digested sludge, wastewater effluent, and biosolids samples.[5,15-17,19,20] Parameters such as geographical regions, wastewater characteristics (e.g., pH, dissolved oxygen, conductivity), wastewater treatment plant (WWTP) operations and processes (e.g., stages of treatment sampled and types of treatment), and environmental conditions are factors contributing to the microbial community's diversity and abundances of individual species.[16,21] It is possible that these traditional and emerging methods can be integrated, identifying the nucleic acids of unique and dominant microorganisms present in biosolids that can lead to the development of novel markers. The presence of biosolid markers in the environment can help indicate potential off-site

migration of biosolids material from land-applied fields but cannot necessarily indicate that pathogenic microorganisms are viable and transmitted in the environment; posing public health risks. Additional studies would need to be conducted to assess any potential health risks that may be associated with pollutants of biosolids.

Specific Research and Objectives and Rationale

The overall goal of this research is to evaluate the fate and transport of biosolids in surface waters following land application through the development and validation of novel biosolid microbial markers. This research fills a critical knowledge gap by providing data to begin assessing the potential environmental impacts associated with the land application of biosolids. Additionally, this research may enhance the evaluation of current environmental waste management regulations and policies by providing tools to wastewater treatment plants (WWTPs) and the United States Environmental Protection Agency (USEPA) for best biosolids management practices. The model for this dissertation is described in Figure 2. Specifically, this dissertation examined the following aims:

Aim 1: To evaluate current knowledge of microbial occurrence following land application of biosolids in the environment. A systematic literature review was conducted to analyze occurrence of microorganisms in field studies. The review helped determine the types of studies conducted, the microbial indicators researched, and if microorganisms analyzed were linked to biosolids as the source of pollution through three primary routes of exposure (air, water, and soil).

Aim 2: To identify and validate candidate microbial biosolid markers for detection in environmental water samples. High-throughput pyrosequencing was used to examine the microbial communities present in biosolid samples for the development of novel biosolid microbial markers. These candidate biosolid markers were evaluated (e.g., *in silico* and in environmental samples) for sensitivity and specificity to biosolids. The pyrosequencing approach allowed deep sequencing of the samples, including microorganisms that cannot be (or have not yet been) cultured and are not usually monitored. Markers with the highest sensitivity and specificity were selected for a field study (Aim 3).

Aim 3: To conduct a field study on the environmental detection of candidate microbial biosolids markers (from Aim 2) in surface waters potentially impacted by land-applied biosolids. Using microbial source tracking (MST) techniques, surface waters near biosolids' land application fields were tested to demonstrate environmental detection of the following microorganisms: fecal indicators, human-specific microbial markers, and candidate biosolid microbial markers (identified in Aim 2). These markers were compared based on their ability to be detected in surface waters following land application of biosolids.

Scientific Significance

This research contributes scientific support needed to better characterize microbial occurrence in an exposure pathway (surface water) associated with land application of biosolids. To the best of our knowledge, this study represents the first utilization of MST techniques to detect biosolid microbial markers in water sources following land application of biosolids. Given advances in pyrosequencing and

molecular detection technologies, candidate microbial markers associated with biosolids were developed and applied in the water environment. Additionally, the approach and knowledge resulting from this project could eventually help link contaminant sources to impacts and aid in designing preventive or remedial strategies.

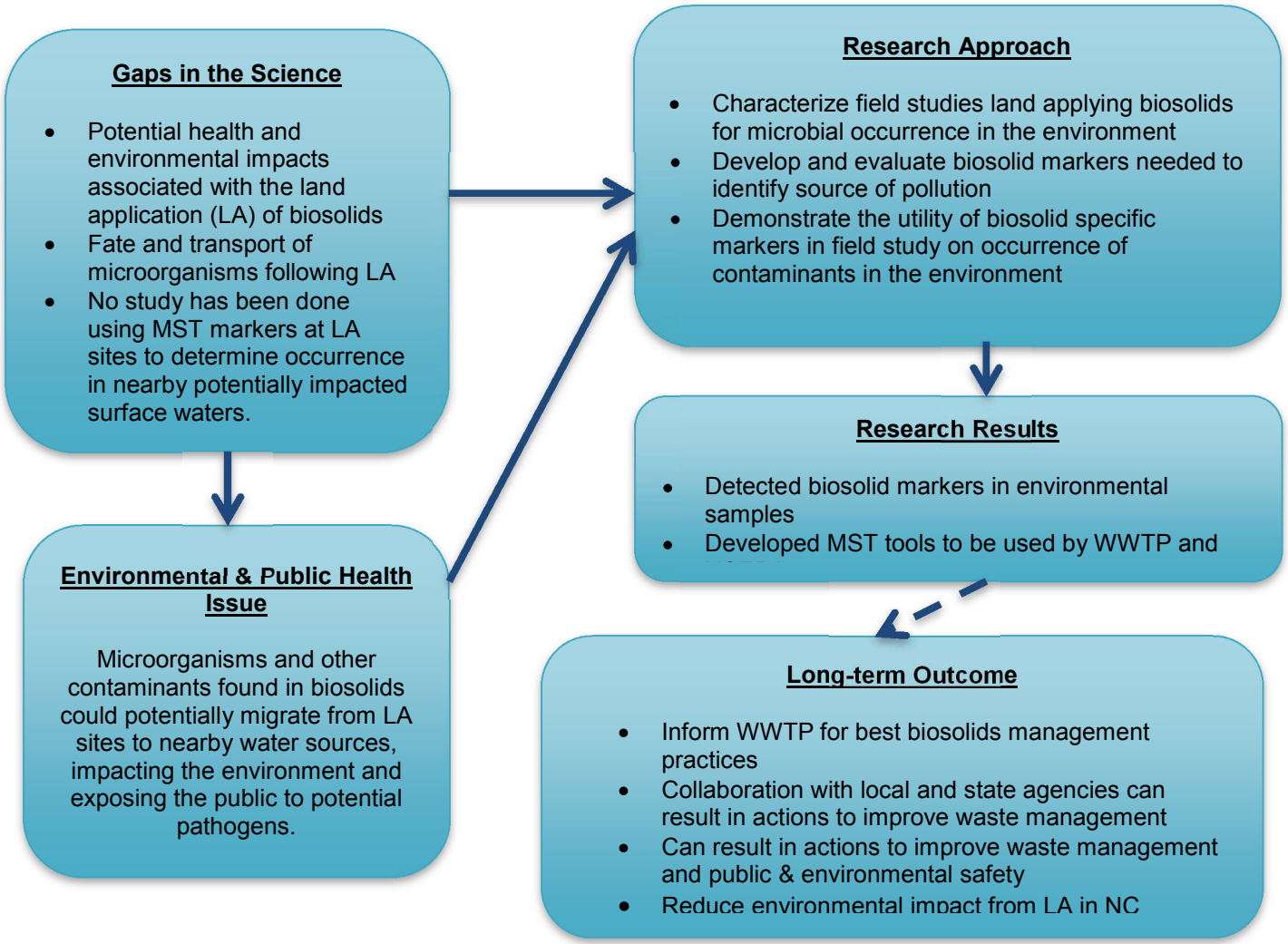

Figure 2. Dissertation research model.

CHAPTER 2. A SYSTEMATIC REVIEW OF MICROBIAL OCCURANCE FOLLOWING LAND APPLICATION OF BIOSOLIDS

Summary

Land application of biosolids is a method for disposal of treated human waste. Despite common use in waste management, public controversy regarding safety suggests the importance of synthesizing evidence regarding impacts on environmental quality. Biosolids applied to land could potentially harbor microorganisms capable of surviving in the environment and being transported off-site via air, soil, and/or water. To gain insight on microbial occurrence following land application of biosolids, we conducted a systematic review of published studies analyzing microorganisms detected in the environment following land application. The search was conducted on August 12, 2014, using four databases and the following three keywords and their derivatives: “biosolids,” “microorganism,” and “land application.” Twenty-eight studies met our inclusion criteria that investigated occurrence of microorganisms following land application. Our conclusion, based on these studies, is that there is evidence of microbial detection in the environment but traditional fecal indicators (used most often for assessment) cannot distinguish whether the source of pollution is associated with biosolids. Therefore, non-traditional markers are better for tracking and identifying sources of potential biosolid pollution.

Introduction

Biosolids are the solids generated during the treatment of human sewage. Upon proper treatment they can be applied on agricultural land as fertilizer. The land application of biosolids is an approved method for disposal of treated human waste, defined by the U.S. Environmental Protection Agency (USEPA) as "the spreading, spraying, injection, or incorporation of sewage sludge, including a material derived from sewage sludge (e.g., compost and pelletized sewage sludge), onto or below the surface of the land to take advantage of the soil enhancing qualities of the sewage sludge."[22] The treatment process and the level of pathogen or fecal indicator concentrations determine the classification and the usage of biosolids at land application sites. Class A biosolids have a more stringent treatment process resulting in a higher reduction of pathogens and fecal indicator organism density,[1] therefore, they can be land-applied with no restrictions and/or sold to the public as compost. Class B biosolids are produced from treatment processes that result in higher fecal indicator concentrations and pathogens,[1] therefore, Class B biosolids require restriction from use on fields for food crops, for grazing animals, and where there is public contact. These restrictions are important because the majority of biosolids that are land-applied in the United States are Class B biosolids.[2]

The application of biosolids has reduced the amount of sewage sludge that would go into landfills or incinerators; approximately 60% of the 5.6 million dry tons of sewage sludge disposed of annually in the United States is land-applied.[2] According to the Clean Water Act Amendments of 1987, the EPA was required to

implement regulations that monitor and control treatment and management practices of land application of biosolids, resulting in the Part 503 Rule.[1,22]

Despite land application being a commonly used and regulated method for waste management, controversy remains about the advantages and disadvantages of land application of biosolids as it pertains to health and environmental impact. Biosolids could potentially harbor microorganisms, raising concerns for communities near sites receiving land-applied biosolids [2,8,23,24]if these microorganisms are capable of surviving and migrating off-site following land application. To gain insight into the current state of science that investigates microbial occurrence following land application of biosolids, we conducted a systematic review of field studies tracking microbial contaminants in the environment following land application to better understand the impact this method of waste disposal has on the environment. This systematic review synthesizes the results of field studies that analyzed various microorganisms off-site following the land application of biosolids through three primary routes of potential environmental exposure (air, water, and soil).

Materials and Methods

Data Sources and Search Strategy

Four databases were searched for peer-reviewed research articles (Embase, Environmental Science and Pollution Management via EBSCO, Institute for Scientific Information (ISI) Web of Science, and PubMed) without date or geographical restrictions. Three domains of keywords, “biosolids” AND “microbes” AND “land application,” were used to iteratively create synonymous search terms

until no improvements in the search were identified.[a] The search was conducted August 12, 2014, with slight modifications for database compatibility. Additional articles were included from citations in the articles found through the database searches.

Study Screening

After removing duplicates, two authors (author 1: PMP, author 2: JGL) independently coded the abstracts and titles of records for inclusion/exclusion. Commentaries, reviews, non–peer-reviewed literature, government agency reports, thesis/dissertations, conference abstracts, and legal documents were excluded. Inclusion criteria included articles that specifically discussed the presence of microorganisms (e.g., viruses, bacteria, helminthes, and protozoa) following the land application of biosolids (biosolids defined as treated sewage sludge). Studies that only measured chemicals, metals, personal care products, nutrients, food crops, treatment/management of waste, or animal waste were excluded. Figure 3, based on PRISMA guidelines,[25]shows the inclusion process. The coders reviewed and discussed discrepancies until an agreement was reached on all records.

Data Extraction

One author read each identified study and included in an evidence table (Table 1) the following information: the microorganism(s) analyzed and if the

[a] **Example search string:** *(((Biosolids OR biosolid OR "refuse disposal" OR treated sewage OR "waste management" OR fertilizers OR sewage treatment OR treatment of sewage OR treating sewage OR sewage sludge)* ***AND*** *("land application" OR "land-applied" OR "land spreading" OR landspreading OR "land-spreading" OR "Part 503")* ***AND*** *(Microbe OR microbes OR pathogen OR pathogens OR microbiology OR (bacteria AND pathogenicity) OR (viruses AND pathogenicity) OR "microbial viability" OR (bacteria AND isolation) OR (bacteria AND purification) OR (viruses AND purification) OR (viruses AND isolation) OR microorganism)))*

microorganism(s) was able to be detected in the environment; the environmental testing conditions (e.g., biosolids application loading sites, downwind following high wind events, depths of soil or water testing); the environmental medium (e.g., air, soil, and/or water); the geographical location; the type of biosolid and application method; and if there was a method of linking the microorganism(s) to biosolids as a potential source of pollution.

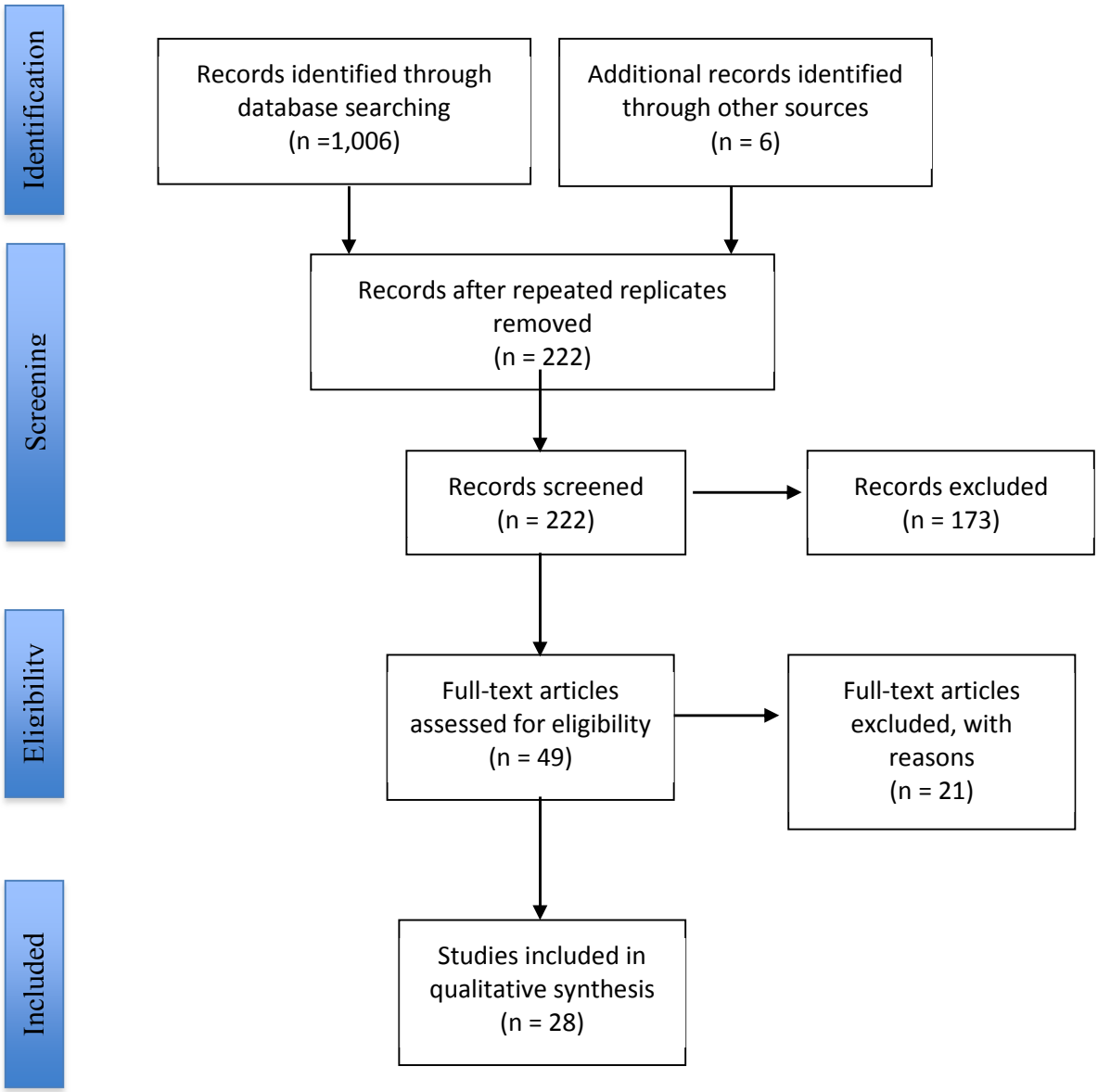

Figure 3. PRISMA diagram for systematic review. [25]

Results and Discussion

We identified 28 studies meeting our inclusion criteria (Table 1). Of these studies, 14 sampled bioaerosols,[8,9,26-37] ten sampled soil,[38-44] three sampled water,[45-47] and one sampled water and soil.[48] Figure 3, based on PRISMA guidelines, shows the inclusion process. The literature suggests that there is the potential for microorganisms to travel off-site in the environment when biosolids are applied on agricultural fields. All studies identified microorganisms through at least one of three primary routes of exposure (air, soil, and water) during/following land application of biosolids. Of all the studies in this review, 60% analyzed the occurrence of microbial indicators, 18% analyzed pathogens, and 22% analyzed other microbes. The majority of the studies were conducted in arid, dry conditions in the Southwestern part of the United States. All biosolids in the studies were anaerobically digested (dewatered cake or liquid) with the exception of three that additionally analyzed Class A,[29] aerobically digested,[41] or thermally dried digested sludge/composted sludge.[40]

Table 1.

Summary of 28 Peer-Reviewed Field Studies That Have Evaluated the Occurrence of Microorganisms Following Land Application of Biosolids

Study	Microorganism	Environmentally detected	Conditions tested	Measure	Geographical location	Biosolid Type	Application Method	Biosolids associated
Sorber et al 1984	Total coliforms	Y	at 50m downwind	bioaerosols	Four unidentified sites	Liquid - anaerobically digested	sprayed on fields	N
	Fecal coliforms	Y						
	Fecal streptococci	Y						
	Enterovirus	N						
Bitton et al., 1984	Poliovirus 1	Y	Summer months - soil cores (exposed to natural conditions) treated with virus seeded liquid sludge to test for survival: up to 35 days	soil	Pensacola, FL	Aerobically and anaerobically digested	NA	N
	Poliovirus 1	N	Summer months - leachate from the virus seeded liquid sludge to test for survival, soil cores (exposed to natural conditions)					
	Poliovirus 1	N	fall/winter months - soil cores (exposed to natural conditions) treated with virus seeded liquid sludge: inactivated between days 8 and 21 and in leachates					
	Echovirus 1							
Pepper et al., 1993	Fecal streptococci	Y	soil migration field and lab study (microcosms) - evaluate survival and vertical transport under the following coniditoions detected: between 150-200 cm in depth, only before 84 days post injection for fall but 50 days for spring, and increases in numbers following rainfall	soil	Tucson, AZ	Mesophilic anaerobically digested	liquid injection onto agricultural land	N
	Fecal coliforms	Y						
	Total coliforms	Y						

Study	Microorganism	Environmentally detected	Conditions tested	Measure	Geographical location	Biosolid Type	Application Method	Biosolids associated
Pillai et al 1996	Heterotropic bacteria	Y	only at the "Hopper" Loading sies	bioaerosols	Sierra Blanca, TX	Dewatered - anaerobically digested	surface applied with mechanical spreaders (hoppers) as semi solid "cake" product	Y
	Clostridium spp	Y						
	Salmonella spp	N	Upwind, interface between sludge appication site and population center, application site, hopper loading sites, and old application sites					
	Fecal coliforms	N						
	Coliphages	N						
Dowd et al., 1997	Heterotropic bacteria	Y	Background sites	bioaerosols	Sierra Blanca, TX	Dewatered - anaerobically digested	surface applied with mechanical spreaders as semi solid "cake" product	N
	Salmonella spp	N						
	Bacteriophages	N						
	Thermotolerant clostridia	N						
	Fecal streptococci	N						
	Total coliforms	N						
	H2S producers	N						
	Fecal coliforms	N						
	Salmonella spp	Y	Loading sites					
	Bacteriophages	Y						
	Thermotolerant clostridia	Y						
	Fecal streptococci	Y						
	H2S producers	Y						
	Total coliforms	N						
	Fecal coliforms	N						
	Salmonella spp	Y	Application sites					
	Bacteriophages	Y						
	Thermotolerant clostridia	Y						
	Total coliforms	Y						
	Fecal coliforms	Y						
	Fecal streptococci	N						
	H2S producers	N						
Dowd & Pillai., 1999	Clostridia	Y	Loading sites	bioaerosols	Sierra Blanca, TX	Dewatered - anaerobically digested	surface applied with mechanical spreaders as semi solid "cake" product	Y
Lang et al., 2003	*E. coli*	Y	unamended and biosolid amended soils (conventionally and enhanced treated)	soil	Ascot, UK	dewatered anaerobically digested sludge (DMAD), composted sludge (CPT), thermally dried digested product (TDD)	in small plots by hand and incorporated immediately into soil 100mm depth using a cultivator	N

Study	Microorganism	Environmentally detected	Conditions tested	Measure	Geographical location	Biosolid Type	Application Method	Biosolids associated
Rusin et al., 2003b	*Staphylococcus aureus*	N	during land application and within 2m of application site	bioaerosols	15 locations - SW to E USA	Class A and Class B	sprayed and spread on fields	N
Brooks et al., 2004	*E. coli*	N	Transport model: aerosolize seeded groundwater with *E. coli* and coliphage (MS-2) monitoring bioaerosols from similar liquid biosolids sprayer at downwind distances from 2 to 70 m	bioaerosols	Not specified but group is in AZ	NA (seeded water experiment to mimic biosolids liquid application)	liquid biosolids spray applicator/tanker	N
	Coliphages - MS-2	Y						
Brooks et al., 2004	Total coliforms	Y	only during high humidity (>10%) within 3m of operation	bioaerosols	Tucson, AZ	Anaerobically digested liquid - Class B	liquid biosolids spray applicator/tanker	N
	E. coli	Y						
	C. perfringens	Y	at various environmental conditions (i.e. extreme heat, relative humidity, and UV levels) in bioaerosols					
	Aspergillus spp	N	biosolids and bioaerosols within 3m of operation					
	Coliphages	Y	in bioaerosol samples within 3m of operation					
	Heterotropic bacteria	Y	aerosolized the most at application sites most likely due to aersolised soil					
	Norwalk-like viruses	N	biosolids and bioaerosols					
	Enterovirus	N						

Study	Microorganism	Environmentally detected	Conditions tested	Measure	Geographical location	Biosolid Type	Application Method	Biosolids associated
Brooks et al., 2005	Total coliforms	N	as aerosols collected from either downwind or upwind (background) samples	bioaerosols	10 sites across: AZ, CA, WA, VA, TX, IL	Anaerobic Class B cake (mostly) and liquid biosolids	spreading, slinging, and spray applications	N
	E. coli	N						
	C. perfringens	N						
	Hepatitis A	N						
	Coliphages	N						
	Enterovirus	N						
	Heterotropic bacteria	Y	readily detected with the exception of sites located in areas of high relative humidity where soils were moist and when soil was not incorporated into the biosolids loading					
	Norovirus	Y	only in one site from study were three samples detected (during slinging (at 5m) and loading (at 2m) conditions)					
	Total coliforms	Y	within 15m of biosolid sites only during loading operations					
	E. coli	Y						
	C. perfringens	Y	within 15m distances during land application and loading events					
Zaleski et al., 2005	Fecal coliforms	Y	Field -concrete drying beds under various environmental conditions (e.g. rainfall, animals)	soil	Tucson, AZ	Mesophilic anaerobically and aerobically digested Class B biosolids	concrete solar drying beds	N
	Salmonella spp							
	Heterotropic bacteria							
	Ascaris spp	N						
	Enterovirus							
	Fecal coliforms	Y	Lab - in biosolids and biosolid amended soil (drying and rewetting conditions)					
	Salmonella spp	N						
	Heterotropic bacteria	Y						
Tanner et al., 2005	Coliphages	N	during land application of liquid Class B biosolids; samples taken downwind 2m	bioaerosols	Tucson, AZ	Liquid Class B - Mesophilic anaerobic digestion and groundwater seeded with E. coli and MS2 coliphage (for comparison)	liquid biosolids spray applicator/tanker (used for both biosolids and seeded groundwater)	N
	Total coliforms	N						
	E. coli	Y	greater aerosolization during land application of seeded groundwater than during land application; samples taken downwind 2m					
	MS2 Coliphage	Y						

Study	Microorganism	Environmentally detected	Conditions tested	Measure	Geographical location	Biosolid Type	Application Method	Biosolids associated
Paez-Rubio et al., 2006	Total bacteria	Y	soil/biosolids composite, aerosols (biosolids disking and control disking)	bioaerosols	Southwest of Phoenix, AZ	Class B, Mesophilic anaerobic digestion dewatered biosolids	spread on fields and then disk incorporated within 48hrs after spreading into soil	N
	Heterotropic bacteria	Y						
	Total coliforms	Y						
	Clostridia	Y						
	endotoxin	Y						
	Total bacteria	Y	soil samples					
	Heterotropic bacteria	Y						
	Total coliforms	N						
	Clostridia	Y						
	endotoxin	Y						
Baertsch et al., 2007	*Clostridia (C. bifermentans)*	N	Downwind: low wind events (<2 m/s): "no disking - aerosol samples were collected upwind and downwind of fields which biosolids had been applied withing the previous 36hrs"	bioaerosols	Southwest of Buckeye, AZ	Dewatered Class B anaerobically mesophilically digested	spread on fields and then disk incorporated within 36hrs of sampling	Y
	Chloroflexi sp	Y						
	Euryarchaeota	Y						
	Clostridia (C. bifermentans)	Y	Downwind: high wind events (>5 m/s): "disking - aerosol samples collected downwind of land applied fields where previously applied biosolids were disk incorporaed within 36hrs of sampling"					
	Chloroflexi sp	Y						
	Euryarchaeota	Y						
	Clostridia (C. bifermentans)	N	Upwind: high (>5 m/s) and low wind (<2 m/s) events during disking and no disking					
	Chloroflexi sp	N						
	Euryarchaeota	N						
	Clostridia	Y	Downwind: high wind events (>5 m/s): "no disking - aerosol samples collected downwind of fields on which biosolids had been applied withing the previous 36hrs"					
	Chloroflexi	Y						
	Euryarchaeota	Y						

Study	Microorganism	Environmentally detected	Conditions tested	Measure	Geographical location	Biosolid Type	Application Method	Biosolids associated
Paez-Rubio et al., 2007	Total bacteria	Y	aerosols are emitted during spreading of dewatered Class B biosolids	bioaerosols	Phoenix, AZ	Class B, Mesophilic anaerobic digestion dewatered biosolids	spread onto fields using a side discharge slinger	N
	Heterotropic bacteria	Y						
	Total coliforms	Y						
	Sulfite-reducing *Clostridia*	Y						
	endotoxin	Y						
Brooks et al., 2007	*Actinobacteria*	Y	aerosolized samples 5m downwind of biosolids and soil operation sites; background (upwind)aerosol samples collected (20min prior to application and without disturbance of site), and soil samples.	bioaerosols	South Central Arizona	Mesophilic anaerobically digested Class B biosolids	loading and slinging biosolids land application operations occurred	Y
	alpha - and *beta-proteobacteria*	Y						
	Firmicutes	Y						
	Chloroflexi	N						
	Bacteroidetes	Y	aerosols downwind biosolids and soil operation sites, soil samples					
	gamma proteobacteria	Y	aerosols downwind and background operations, soil samples					
	Actinobacteria	N	biosolid samples directly from preapplied loads					
	alpha, beta, gamma-proteobacteria	Y						
	Chloroflexi	Y						
Brooks et al., 2007	endotoxin	N	No increase in percentage/concentration due to biosolids	soil	Tucson, AZ; CA, NH, and WA	Liquid - anaerobically digested - Class B	injector applicator approximately 15 cm below soil	N
	antibiotic resistant bacteria	N						

Study	Microorganism	Environmentally detected	Conditions tested	Measure	Geographical location	Biosolid Type	Application Method	Biosolids associated
Lang et al., 2007	*E. coli*	Y	in dewatered mesophilic anaerobically digested, enhanced treated biosolids, control soil or sludge-amended soil	soil	Ascot, UK	conventional treated: Dewatered mesophilic anaerobically digested sludge (DMAD), enhanced treated: thermally dried digested sludge (TDD) and composted sludge	by hand and incorporaed to a depth of 10cm with a pedestrian operated rotart cultivator	N
	Salmonella	N	in dewatered mesophilic anaerobically digested, enhanced treated biosolids, control soil or sludge-amended soil					
	F-specific RNA bacteriophage	N	in control soil, sludge-amended soil, and enhanced treated biosolids					
	F-specific RNA bacteriophage	Y	in dewatered mesophilic anaerobically digested-detected in low numbers					
Lang and Smith, 2007	*E. coli*	Y	Model incubation study - soil (controlled plots - sandy loam and silty clay) collected to a depth of 10 cm; increase in numbers in biosolid amended soils vs control non ameneded soils and observed decline with time in both soil plots	soil	Ascot, UK	Dewatered mesophilic anaerobically digested (DMAD)	random grab composite samples incorporated at depth of 10cm to soil plots	N
Lapen et al., 2008	*E. coli*	Y	in groundwater 1.2m and 2m depth, observed pre application and immediately after application; in biosolid amended soils with both application methods	groundwater	Winchester Ontario, Canada	Liquid - anaerobically digested	two different methods: surface spreading followed by incorporation using a cultivator and a one-pass AerWaySSD slurry deposition system (applies above surface to avoid tillage)	N
	Clostridium perfringens	Y	in biosolid amended soils with both application method and in ground water at 1.2 and 2.0 m post application					
	Clostridium perfringens	N	in control soil and in any 1.2 or 2.0 m ground water samples pre application					

Study	Microorganism	Environmentally detected	Conditions tested	Measure	Geographical location	Biosolid Type	Application Method	Biosolids associated
Surampalli et al., 2008	Fecal coliforms	N	biosolid-amended soil from surface (0-5 cm) and deep layers (20-25 cm), groundwater (well samples), and background soil samples (no biosolids applied)	water and soil	University of Missouri	Class B	Injected into soil surface	N
	Fecal streptococci	N						
Tanner et al., 2008	Coliform bacteria	Y	when biosolids were applied to land or loaded into land applicators	bioaerosols	NV, WA, DC, CA, IL, AZ	Class B - anaerobically digested	biosolids spread by slinger, spray gun, spreader, and splash plate (spray tanker)	N
	Coliform bacteria	N	in air samples collected: directly downwind of biosolids piles, downwind of splash plates, or in background samples collected away from or before application					
	Coliphages	N						
	Coliphages	Y	few bioaerosol samples and only when biosolids were loaded into applicators					
	Heterotropic bacteria	Y	regardless if biosolids were applied					
Gottschall et al., 2009	*E. coli*	Y	both application methods: pre and post applications at 1.2 m and 2.0 m depths; <100 and >100 days post application; post application rainfall to at least 1.2 m depth below the surface	groundwater	Winchester Ontario, Canada	Dewatered - anaerobically digested	surface spreading with shallow incorportaion and direct injection into top soil (~0.1 m depth)	N
	Enterococci	Y						
	Clostridium perfringens	Y						

Study	Microorganism	Environmentally detected	Conditions tested	Measure	Geographical location	Biosolid Type	Application Method	Biosolids associated
Zerzghi et al., 2010	Heterotropic bacteria	Y	in both control soil and amended soil samples	soil	Tucson, AZ	Anaerobically digested liquid Class B	injected into soil surface (0-30 cm) with a semi trailer applicator	N
	Coliphages	N	in soil samples collected 10 mo after last application (control and amended soil)					
	Salmonella							
	Enterovirus							
	Total coliforms	Y	in biosolid amended soils (collected 10 mo after last application)					
	Fecal coliforms	Y						
	antibiotic resistant bacteria	Y	in biosolid amended soils (collected 10 mo after last application) and unamended soil					
Zerzghi et al., 2010	Proteobacteria	Y	surface (0-30 cm) unamended and biosolids-amended soil samples	soil	Tucson, AZ	Anaerobically digested liquid Class B	injected into soil surface (0-30 cm) with a semi trailer applicator	N
	Actinobacteria	Y						
	Acidobacteria	Y						
	Firmicutes	Y						
	Bacteroidetes	Y						
Esseili et al., 2012	*E. coli*	Y	offsite transmission under heavy rain events	water	NW Ohio	Class B - liquid slurry	subsurface injection	Y

Study	Microorganism	Environmentally detected	Conditions tested	Measure	Geographical location	Biosolid Type	Application Method	Biosolids associated
Zerzghi et al., 2010	Heterotropic bacteria	Y	in both control soil and amended soil samples	soil	Tucson, AZ	Anaerobically digested liquid Class B	injected into soil surface (0-30 cm) with a semi trailer applicator	N
	Coliphages	N	in soil samples collected 10 mo after last application (control and amended soil)					
	Salmonella							
	Enterovirus							
	Total coliforms	Y	in biosolid amended soils (collected 10 mo after last application)					
	Fecal coliforms	Y						
	antibiotic resistant bacteria	Y	in biosolid amended soils (collected 10 mo after last application) and unamended soil					
Zerzghi et al., 2010	Proteobacteria	Y	surface (0-30 cm) unamended and biosolids-amended soil samples	soil	Tucson, AZ	Anaerobically digested liquid Class B	injected into soil surface (0-30 cm) with a semi trailer applicator	N
	Actinobacteria	Y						
	Acidobacteria	Y						
	Firmicutes	Y						
	Bacteroidetes	Y						
Esseili et al., 2012	*E. coli*	Y	offsite transmission under heavy rain events	water	NW Ohio	Class B - liquid slurry	subsurface injection	Y

Air Studies

Fourteen of the 28 studies identified in this systematic review were field studies that analyzed the transport of microorganisms in bioaerosols under various conditions during and following land application of biosolids. With the exception of one study conducted by Sorber et al. (1984) that did not identify sample sites, all others were conducted between two groups of researchers, analyzing land application sites mostly in Arizona and Texas where the climate is hot and arid. Three studies did a comparison between biosolid samples collected from various geographical locations, including Texas and Arizona. [29,36,49] Table 2 lists the microorganisms that were observed in bioaerosols and the majority (38%) of all organisms in this systematic review were indicator organisms, which are typically used to assess fecal pollution. Of the total studies (N=28), 14% directly analyzed the potential for pathogens to be transported via bioaerosols. Most of the studies investigated multiple organisms, giving a total of 57 microorganisms analyzed for potential microbial aerosolization under biosolid land application field conditions.

Two studies demonstrated the ability to detect and track microorganisms in bioaerosols during land application of biosolids back to the original source of pollution, the biosolid piles. [50, 51] All studies, under various conditions (identified in Table 1), were able to measure at least one microorganism with the exception of one study that collected aerosol samples from 15 different sites but did not find occurrence via bioaerosol of the targeted organism *Staphylococcus aureus* [29]

Table 2.

Microorganisms (n=57) Investigated in Bioaerosols Under Various Biosolids Land Application Conditions

(Note. Most studies investigated multiple organisms, total N=96)

Type	Organism	# Studies analyzed organism (n=51)	% Total of microbes measured (N=96)
Indicator	Coliform Bacteria	14	38
	Fecal Streptococci	2	
	Anaerobic Bacteria	7	
	Bacteriophages	6	
	Heterotrophic plate count	7	
Pathogen	*Salmonella spp*	2	9
	Staphylococcus aureus	1	
	Norwalk-like viruses	1	
	Enterovirus	3	
	Hepatitis A	1	
	Norovirus	1	
Other	*C. bifermentans*	1	13
	Chloroflexi sp	2	
	Euryarchaeota	1	
	Actinobacteria	1	
	alpha, beta, gamma-proteobacteria	1	
	Firmicutes	1	
	H2S producers	1	
	Endotoxin	2	
	Total bacteria	2	

Soil Studies

Ten of the studies identified in this systematic review were field studies that analyzed the transport of microorganisms in soils under various conditions during and following land application of biosolids. Rainfall events increased microorganisms present in the soil. [39, 52] Soil can be a protective filter preventing vertical transport,[39-41] which is important for Arizona because it relies on aquifers for its drinking water supply. Similar to the air studies, soil studies, with the exception of one, were all conducted in Arizona under arid and warm conditions. One study investigated the impact of 20-year application and reported no pathogen detection and no impact on the soil microbial community.[43, 44] Table 3 provides a breakdown of all of the microorganisms analyzed in soils.

Table 3.

Microorganisms (n=31) Investigated in Soils Under Various Biosolids Land Application Conditions

(Note: Most studies investigated multiple organisms, total N=96)

Type	Organism	# Studies analyzed organism (n=31)	% Total of microbes measured (N=96)
Indicator	Fecal Streptococci	2	16
	Fecal Coliforms	4	
	Total Coliforms	2	
	Heterotrophic plate count	2	
	E. coli	3	
	F+ coliphages	2	
Pathogen	Poliovirus I	1	7
	Echovirus I	1	
	Salmonella	3	
	Enterovirus	2	
Other	Ascans spp	1	9
	Endotoxin	1	
	ABR/ARB	2	
	Proteobacteria	1	
	Actinobacteria	1	
	Acidobacteria	1	
	Firmicutes	1	
	Bacteroides	1	

Water Studies

Field studies investigating the impact of microbial occurrence via water sources resulted in the fewest number of articles. Of the four reported, all investigated the impact to groundwater. One reported after an 8-10 year study no pathogen contamination.[53] However, two reported that rainfall events contributed to *E. coli* contamination of shallow groundwater following biosolids application.[54,55] A recent study investigated the potential of transport of *E. coli* off-site following the land application of biosolids on agricultural fields and not only concluded this possibility during heavy rain events but also the necessity to develop molecular based methods (i.e., combining enumeration of *E.coli* with genetic fingerprinting) to better identify sources of pollution.[55] The impact of biosolids on surface and groundwater is of concern due to potential of contamination from run-off during rain events.[39,41,54-56] Table 4 lists the microorganisms analyzed in the water environment.

Table 4.

Microorganisms (n=8) Investigated in Water Sources Under Various Biosolids Land Application Conditions

(Note: Most studies investigated multiple organisms, total N=96).

Type	Organism	# Studies analyzed organism (n=8)	% Total of microbes measured (N=96)
Indicator	*E. coli*	3	8
	Enterococci	1	
	Fecal Coliforms	1	
	Fecal Streptococci	1	
	C. perfringens	2	

Main Findings

This systematic review synthesized the literature on field studies detecting microorganisms in the environment when biosolids were land-applied under various conditions. With land application being the primary means for waste disposal, this review provides an overview of the literature analyzing the microorganisms investigated, their capability to be transported off-site, and the impact this practice potentially has on the environment.

The commonality in all of the studies in this systematic review described land application as the primary means of disposing of biosolids and noted that there are potential agricultural benefits. However, these articles also suggest that despite treatment, which reduces the microbial load, microorganisms are present and detection could occur in the environment under certain conditions. Unanswered questions remain pertaining to the frequency, timing, and conditions conducive to off-site microbial detection. Additional limitations include the following: 1) The majority of the studies were conducted by two research groups between two states (Arizona and Texas) in the Southwestern region of the United States; 2) The typical environmental conditions were arid, hot temperatures, limited precipitation, low humidities, and high wind velocities; 3) The majority of the biosolids land-applied were Class B; 4) Traditional microbial indicators were analyzed most frequently; and 5) The majority of the studies conducted involved transport via bioaerosols. Additional findings are categorized and discussed next to address major themes of interest.

Microorganisms Analyzed and The Ability to Link to Biosolids

The majority of the studies identified in this systematic review investigated traditional indicators such as fecal coliforms, total coliforms, enterococcus, and coliphages. These indicators are widely used and considered in predicting fecal pollution, determining water quality, assessing risks to public health associated with exposure, and designing remediation plans. However, there are concerns about solely using these microorganisms as confirmatory indicators of fecal pollution because these indicators are not source-specific.

Six of the 28 studies evaluated in this review investigated novel indicators using molecular methods to identify genetic signatures of microorganisms unique to biosolids. [8,41,42,45,50,51] These studies were successful in tracking microorganisms in the environment, linking them to biosolids as the original source of pollution. Evidence supports the potential feasibility of using these types of indicators as potential biosolid source tracking markers.

A recent review of land-applied waste products suggests that a better assessment of specific pathogens and their transport, regrowth, and survival during land application is needed to ensure the safety of our water and food supplies.[57] However, what seems to be of pubic concern is the potential exposure to pathogens resulting from the land application of biosolids.[6-8,30,58]

In Texas, at one of the largest land application sites in the United States, several studies were conducted that investigated aerosolized microorganisms (e.g., fecal coliforms, total coliforms, male-specific coliphages, hydrogen sulfide producers, fecal streptococci, *Salmonella sp*., and thermotolerant clostridia) following biosolids

application. Traditional microorganisms (e.g., fecal coliforms, total coliforms, male-specific coliphages, fecal streptococci, *Salmonella sp*.) have been utilized as indicators for contamination; however, they are not capable of distinguishing sources of fecal pollution. Additionally, they were not always detected in aerosol samples despite being detected in biosolids.[6,7] Based on this, researchers suggest that these indicators are not capable of surviving aerosolization and other environmental stressors such as UV and temperature extremes.[6,7,26,30]

A study that compared the detection of coliphages and total coliform bacteria in aerosols between the application of biosolids and seeded groundwater inoculated with similar concentrations of those microorganisms concluded there might be properties of biosolids that impact the ability of microorganisms to become aerosolized.[32] There is also research that suggests that bacteria and viruses could adsorb to solid particles, which could prevent aerosolization as well.[30,32,59] These studies concluded no risks to nearby communities based on their findings; however, they did not analyze pathogens and infectivity was not determined in their studies. There are a number of factors that discourage the continual use of only traditional indicators and a few studies have begun to investigate novel approaches, demonstrating the utility of non-traditional microorganisms.

One early study utilizing non-traditional indicators measured hydrogen sulfide–producing bacteria and *clostridia*[8] and suggested that *clostridia* might be a better, more accurate indicator of biosolid contamination because they can survive anaerobic digestion, aerosolization, and other environmental stressors.[6-8] Another study developed and investigated three sensitive and specific biosolids

microorganisms: *Clostridium bifermentans, Chloroflexi,* and *Euryarchaeota.*[9] These indicators were found in 100% of biosolid samples and in less than 11% of soil samples.[9] These studies supplemented each other. Dowd and Pillai (1999) used a method that targeted the 16S-23S interspacer region for DNA fingerprinting, identifying *clostridia* and demonstrating that it could be traced back to its original biosolids piles.[6-8] This method depends on the ability of a microorganism to be cultured, whereas Baertsch (2007) developed non-culture–dependent methods for microbial source tracking of *Chloroflexi* and *Euryarchaeota.*[9]

Potential Pathogen Exposure Following Land Application

Biosolids potentially contain pathogenic microorganisms but the potential for pathogen exposure (via inhalation, ingestion, and direct contact) or adverse health outcomes among human populations in close proximity to land application sites are not well characterized. In places like Tucson, Arizona (where the majority of the studies in the review were conducted), that rely on underground aquifers to supply drinking water, it is important to assess the public's risk of exposure and to investigate the potential for migration through soil.[39] Of the 28 studies, 14 investigated pathogenic microorganisms and under certain conditions were successful in detecting these organisms during or following land application of biosolids (Table 1). Despite these findings, there are still limited data that directly link pathogenic microorganisms to biosolids as the primary source of pollution. According to a report by the NRC (2002), evidence is needed to link adverse health effects to land application of biosolids. Epidemiological studies are limited and challenging to conduct but "absence of evidence is not evidence of absence".[60] In an

attempt to safeguard the public and reduce potential exposure to pathogens present in biosolids, multiple barriers are in place, which include: treatment to reduce the microbial load; restrictions (e.g., distance to water bodies and frequency of applications) and guidelines for land use (e.g., harvesting and grazing animals); and a final environmental barrier is the natural decay over a period of time.[40]

Survival, Regrowth, and Off-Site Migration

One important factor to consider when investigating the impact land-application of biosolids has on the environment is the ability of microorganisms to survive during and following land application. Biosolids are generally land-applied via spraying or spreading onto fields. During such time, trucks are loaded with biosolid materials and either mechanically spread or sprayed and then incorporated into the soil. These mechanical processes (loading and applying) could potentially contribute to the aerosolization of microorganisms due to the disturbance of the biosolids and the soil that naturally harbors microorganisms.[8,23,50,61] Most microorganisms capable of surviving the treatment process then undergo environmental stressors that inactivate them during bioaerosol transport.[32,61]

A comparison study of application processes suggests that during liquid spraying, microorganisms get trapped in the heavy large droplets and fall to the ground, limiting aerosolization of organisms.[49] However, the dewatered cake product that is land-applied can concentrate microorganisms[47] and contribute to the adsorption of microorganisms to the solid particles. Viruses are known to adsorb to solids, which could aid in their survival; however, this adsorption contributes to their inability to be transported through the soil as indicated by the monitoring of leachate,

where no viruses were detected during one seasonal study.[38] Soil can be a protective filter preventing vertical transport.[39-41] Soil studies concluded that under favorable environmental conditions (e.g., increases in soil moisture following rainfall or the introduction of animal fecal contamination), microorganisms could possibly survive, regrow, recolonize, and migrate off-site.[41] Trends were observed in some review articles using indicator organisms introduced in soil via biosolids application that concluded soil properties affect survival and regrowth—microbial increases were observed with increasing soil moisture, decreasing soil temperature, and with finer-textured soils.[41]

Zaleski et al. (2005) conducted a lab and field study using a solar drying bed for indicator and pathogen reduction to determine if these microorganisms were capable of re-growing in biosolids and biosolid-amended soils. The survival of pathogens was investigated by comparing regrowth of *Salmonella* under lab and field conditions and results showed that *Salmonella* were not able to survive and regrow in biosolids or in biosolids-amended soil under controlled lab conditions.[41] However, under field conditions following rainfall, increases in *Salmonella* numbers were observed from the original biosolids samples to those tested in biosolid-amended soils. Confirmatory serotyping distinguished *Salmonella* types and concluded that increases were due to recolonization of pathogens to biosolids by confounding factors such as animal fecal pollution and did not match any found in biosolids.[41] Additionally, viruses may survive under certain favorable conditions in biosolid-amended soils versus being inactivated in warm and dry seasons.[38] According to Bitton (1984), heavy rain events could contribute to virus survival due

to the moist soil; and the authors concluded that virus survival in biosolid-amended soil is impacted by aridness and temperature.

Impact of Land Application

The majority of the studies included in this systematic review were conducted immediately following land application of biosolids, making it more challenging to fully understand the impact that long-term application has on environment quality. Some studies concluded that the application of biosolids results in an increase in microorganisms immediately following application, but within the short study times, the microbial community returned to similar compositions as the original soil.[39,40,62,63] However, a study conducted over a 20-year land application period made it possible to examine the decay and survival of microorganisms introduced to the soil during land application, comparing non-amended to amended soils.[40,43,44] The microbial community was examined and the dominant phyla were *Proteobacteria, Acidobacteria, Actinobacteria, Bacteroidetes*, and *Firmicutes,* with a higher number of microorganisms in the biosolid-amended soils, which could suggest an increase in bacterial diversity. The DNA was “extracted, amplified using 16S rRNA primers, cloned, and sequenced”.[44] Two species (*Shigella sonnei* and *Escherichia coli*, both potentially pathogenic) were identified in amended soils but not in non-amended soils, suggesting that these potentially pathogenic microbes are possibly introduced in the environment from biosolids.[44] This study concluded that bacterial diversity is not impacted due to land application of biosolids and this practice is sustainable based on microbial composition.[44]

Strengths of the Study

To the best of our knowledge, this is the first systematic review of its kind that analyzed the peer-reviewed literature examining land application of biosolids and the occurrence of microorganisms in the environment. This review summarized and critically discussed the results of 28 articles evaluating the impact of land application of biosolids on environmental quality. It extracted data assessing the current state of literature, specifically investigating biosolid field studies. Finally, this review concluded that based on nucleic acids or culture-based methods, microorganisms are detected under certain environmental conditions following land application of biosolids. The microbial detection may provide useful information to wastewater treatment plants (WWTPs) and the USEPA for management and possible policy changes if the microorganisms can be linked to biosolids as a possible source of pollution.

Limitations and Generalizability

Publication bias could potentially be a limiting factor in conducting this systematic review due to the inclusion of only peer-reviewed articles and the possibility that some researchers would not report negative findings and therefore their work would not be included in the literature. From our literature review, we were able to identify areas in biosolids research in which science could benefit from further field studies that specifically trace microorganisms following land application to nearby water sources. Most of the field studies were conducted in the Southwestern region of the United States where the conditions are hot and arid. These climatic conditions contribute to the soil condition and, therefore, the survival

and occurrence of microorganisms in the environment. The study results might not be generalizable to other regions of the United States, where different environmental parameters could contribute to survival and occurrence of microorganisms following biosolids land application.

Implications for Policy Makers and WWTP Management

Occurrence of microorganisms has been documented under certain conditions following land application of biosolids. From this research, it is better understood that a sensitive and specific biosolids indicator needs to be used that will also aid in tracing any contaminant to its source. Traditional indicators may not be the best at evaluating transport because current microbial indicators are not source-specific. Therefore, the development of novel biosolid-specific indicators could contribute to science and provide a useful tool for the EPA and WWTPs to better regulate and monitor the land application of biosolids.

Acknowledgements

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CHAPTER 3. DEVELOPMENT AND EVALUATION OF MICROBIAL SOURCE TRACKING MARKERS TO TRACK LAND-APPLIED BIOSOLIDS

Summary

Land application of biosolids is the primary means for municipal wastewater treatment plants (WWTPs) to manage and dispose of biosolids. However, more needs to be known about the fate and transport of associated contaminants and the potential impact on the environment and human health. To investigate such impacts it would be helpful to identify microbial markers that could be used to trace biosolid contaminants to their original source, distinguishing biosolid run-off from other sources of pollution. Hence, it was the goal of this research to examine the microbial community present in biosolid samples to develop and evaluate novel biosolid markers that can be used as microbial source tracking markers in the environment. Two sets of biosolid (Class A and Class B) samples were collected from two different WWTPs in the Southeastern region of the United States, representative of common treatment processes and biosolid types for this region. Total community DNA was extracted and high-throughput 454 pyrosequencing was performed to examine the microbial communities (*Archaea* and Bacteria) present in the samples. This approach allowed deep sequencing of the samples, including microorganisms that cannot be (or have not been) cultured and are not usually monitored. Then, microbial markers were designed and evaluated using polymerase chain reaction (PCR) to test for presence/absence of the candidate markers in additional biosolid

samples (ranging in treatment processes producing both Class A and Class B biosolids) and treated animal manure samples (i.e., dairy, swine, and poultry). The combined pyrosequencing and PCR analyses identified several candidates within the *Archaea* and Bacteria kingdoms as potential microbial markers, and upon further *in silico* analysis, we selected sequences within the following genera to target: *Betaproteobacteria, Leptotrichiaceae, Methanosaeta*, and an unclassified uncultured *Archaea.* The validation study confirmed that these candidate biosolid markers are sensitive to biosolids from various treatment processes. However, the markers do not appear to be specific to treated human wastes—they are able to cross-react with treated animal wastes. These microbes are likely selected and enriched through treatment processes. The candidate biosolid markers may still have utility at sites where treated (digested, composted) animal waste is not present, or else in combination with a human-specific marker. This research provides an approach for understanding the potential impact land application of biosolids has on the environment. Traditional microbial indicators (e.g., fecal coliforms, total coliforms) are not source-specific and the development of these novel biosolid indicators could allow the U.S. Environmental Protection Agency (USEPA), state agencies, and wastewater treatment plants to better regulate and monitor the land application of biosolids. Eventually, the approach and knowledge resulting from this project could help link contaminant sources to environmental impacts.

Introduction

Biosolids are generated from the biological and/or chemical treatment of the solid components of human waste. Waste treated with a biological process results in

a biosolids product that has a diverse microbial community.[5] Upon proper treatment, biosolids can be applied on fields, adhering to regulations and guidelines established by the USEPA. This is the primary means of managing and disposing of biosolids.

Traditionally, fecal indicator bacteria (e.g., fecal coliforms, total coliforms) are used to determine fecal pollution in the environment because it is too challenging and impractical to test for all possible pathogens. These indicator bacteria are good indicators for fecal pollution because they are found in high quantities in the gut and feces of warm-blooded animals and they are easy to detect. However, when investigating potential biosolid pollution, fecal indicator bacteria are problematic because they are non-specific to biosolid materials and can be found from other sources of fecal contamination including leaky septic tanks, grazing animals, and birds. It has been also suggested that traditional indicators might not survive anaerobic digestion (a common process used to treat wastewater sludge), aerosolization, and other environmental stressors,[6-8] which could limit their tracking abilities. These factors discourage the continued use of only traditional indicators for investigating microbial transport following land application of biosolids and suggest the need for indicators that can be used to trace a contaminant to its original source, distinguishing biosolid run-off from other sources of pollution. Hence, the goal of this research was to examine the microbial community present in biosolid samples and to develop novel indicators unique and abundant in biosolids that can be markers for tracking pollutants in the environment.

Influent wastewater and biosolid samples were collected from two wastewater treatment plants in the Southeastern United States that used two different treatment

processes to produce biosolids (mesophilic anaerobic digestion and thermophilic anaerobic digestion). Total community DNA was extracted and high-throughput 454 pyrosequencing was performed to examine the microbial communities present in all samples. The use of high-throughput 454 pyrosequencing allowed deep sequencing of biosolid samples, including microorganisms that cannot be (or have not been) cultured and are not usually monitored, aiding in the development of novel microbial indicators. The indicators developed in this phase of our research were then used in a field study using microbial source tracking techniques to detect candidate biosolid microbial markers in nearby surface waters. The results of this field study are reported in Chapter 4.

Materials and Methods

Molecular Analysis of Biosolid Samples for Developing Source Tracking Markers (Figure 2)

Environmental sampling

Influent wastewater and biosolid final products were sampled in April 2013 from two Southeastern wastewater treatment plants (WWTPs) in duplicate in sterile Nalgene bottles. All samples were collected from both plants on the same day and transported to the UNC laboratory and processed upon arrival. Twenty-five milliliters of influent samples were filtered using a 0.22 µm Durapore® (Millipore, Billerica, MA) membrane. The filter paper was immediately placed in the PowerBead tube supplied in MoBio PowerSoil kit (MoBio, Carlsbad, CA, and Thermo Scientific, Wilmington, DE) for DNA extraction. All influent samples were filtered in duplicate, for a total of four filters per plant equaling eight samples filtered total. Biosolids were collected from the same two WWTPs in Nalgene bottles and transported to the lab in an ice-

packed cooler. Upon arrival at the lab, 25 mL of biosolid liquid slurry was centrifuged at 5135 x g at room temperature for 20 min (Sorvall RC-3B Refrigerated Centrifuge). The supernatant was decanted and 0.25 g of the pellet was added to the PowerBead tube for DNA extraction following the protocol provided in the MoBio PowerSoil kit (MoBio, Carlsbad, CA and Thermo Scientific, Wilmington, DE).

DNA extraction, PCR, and pyrosequencing

DNA extraction Total community DNA was extracted in duplicate from the influent and biosolid samples collected from the WWTPs following the protocol provided in the MoBio PowerSoil kit (MoBio, Carlsbad, CA, and Thermo Scientific, Wilmington, DE) and the DNA was quantified using a Thermo Scientific NanoDrop Lite Spectrophotometer (UX-83060-01). DNA extractions were done in duplicate, eight per plant, totaling 16 samples. One set of eight was archived and the other set of eight was used for pyrosequencing.

454 Pyrosequencing: Archaea 16S rRNA gene amplification. Extracted DNA was shipped overnight, on dry ice, to Molecular Research DNA (MRDNA) (www.mrdnalab.com, Shallowater, TX), where all amplification and pyrosequencing was performed for *Archaea.* The primers used for *Archaea* rRNA gene amplification were ARC_349F (GYGCASCAGKCGMGAAW) and ARC_806R (GGACTACVSGGGTATCTAAT) (www.mrdnalab.com, Shallowater, TX). This *Archaea* primer set was selected based on the platform MRDNA used to analyze *Archaea*. The PCR protocol was 94°C for 3 minutes, followed by 28 cycles of 94°C for 30 seconds, 53°C for 40 seconds, and 72°C for 1 minute; with a final elongation step at 72°C for 5 minutes. This amplified a DNA fragment of 457bp length between

the V6 hypervariable regions of the 16S rRNA gene. The samples were assigned oligonucleotide barcodes, which identified each sample individually when mixed together allowing for multiplex sequencing. The barcodes were added between the 454 adaptors and the forward primer by fusing a unique 5-10 nucleotide to the 5' end of the V6 forward primers and the 3' end of the 454 adaptor. After PCR, the amplified products from the different samples were mixed in equal concentrations and purified using Agencourt Ampure beads (Agencourt Bioscience Corporation, MA, USA). The samples were sequenced using a Roche 454 FLX Titanium sequencer (Roche Diagnostics, Branford, CT).

454 Pyrosequencing: Bacterial 16S rRNA gene amplification. DNA was amplified using the FastStart High Fidelity polymerase chain reaction (PCR) system (Roche, Indianapolis, IN). Approximately 25 ng of template DNA was added to each reaction and the following 16S rRNA primers were used for rRNA amplification: Bacteria_577F (AYTGGGYDTAAAGNG) and Bacteria_926R (CCGTCAATTCMTTTRAGT)[64,65] following the PCR protocol 95°C for 2 minutes, followed by 30 cycles of 95°C for 45 seconds, 55°C for 45 seconds, and 72°C for 1 minute, with a final elongation at 72°C for 4 minutes. The Bacteria amplified products were 349bp in length targeting DNA between the V3 and V4 region and were separated on a 1% agarose gel. All PCR reactions were done in triplicate and pooled during gel purification using the Qiagen Gel Extraction Kit (Qiagen, Valencia, CA) and quantified using a Nanodrop Spectrophotometer (Thermo Scientific, Wilmington, DE). The Bacteria samples were then analyzed at the UNC Microbiome

Core Facility using a Roche 454 FLX Titanium sequencer (Roche Diagnostics, Branford, CT).

Pyrosequencing data analysis (Figure 3)

Archaea analysis. For analysis of the *Archaea* data set, the raw barcoded sequence reads were sorted, trimmed, and put through a quality filter using MOTHUR (http://www.mothur.org).[66] Briefly, a pipeline was followed that sorts reads exactly matching the specific barcodes into different samples; trims the reads of the adaptors, barcodes, and primers; and removes any sequences with ambiguous bases or shorter than 200bp length. The sequences were denoised, removing any sequences caused by pyrosequencing errors, and PCR chimeras were removed. Taxonomy analysis was conducted to cluster sequences into operational taxonomic units (OTUs) to compare microbial community groups (99% similarity). From this step we were able to determine the unique and dominant OTUs across all eight samples based on total number of sequences. The 362 OTUs identified were manually searched and only biosolid samples were analyzed. Twenty OTUs were selected (Table 7) and the consensus sequences were determined using a command in MOTHUR at 0.01 cutoff. All consensus sequences (250bp) were blasted using National Center for Biotechnology Information (NCBI) Basic Local Alignment Search Tool (BLAST) using the nonredundant (nr) database. This was done to check isolation sources. If isolation sources were suggestive of animal waste or any other environmental source that was not a result of municipal wastewater treatment it was eliminated (see Table 8 for example of eliminated OTU sequence). If the isolation sources were suggestive of municipal wastewater

treatment processes (Tables 9 and 10), these OTU sequences were selected as possible biosolid markers and additional analyses, including primer design, were conducted (refer to Chapter 4 for primer selection).

Bacteria data analysis. Similar analysis of bacterial data was conducted as described above for *Archaea* using the MOTHUR pipeline with the following exception: The consensus was performed for all sequences at a cutoff of 0.03 and 3,812 OTUs were identified. An analysis of the shared OTUs was created in MOTHUR producing a Venn diagram (Figure 8), illustrating the shared OTUs for biosolids (97% similarity) between both WWTPs (Table 11). The top 10 of the 25 OTUs shared were manually searched as described for Archaea and all other steps were performed in the same manner. Table 12 illustrates an example of an OTU primer set that was eliminated based on undesired (e.g., animal) isolation sources. Tables 13 and 14 show OTUs selected as possible biosolid markers for which additional analyses were conducted (refer to Chapter 4 for primer selection).

Biosolid Primer Development and Performance Evaluation

Biosolid primer selection (Figure 3)

All primer sets selected were ordered and PCR protocols were optimized based on an annealing temperature gradient process. PCR products were then cloned and sequenced to confirm they belonged to the desired microbial target. Products were cloned using a TOPO® TA Cloning kit® for Sequencing with OneShot® TOP10 Chemically Competent *E. coli* (Invitrogen, Carlsbad, CA, USA) according to the protocol provided in the kit. For each library selected, ten colonies were chosen and plasmids were extracted using a QIAprep Spin Miniprep Kit.

Plasmids were then sent to Eton Bioscience (Research Triangle Park, NC) for sequencing confirmation using the M13R as the sequencing primer. Clone sequences were analyzed using NCBI BLASTn (nr database) and isolation sources were analyzed *in silico* to ensure the clones were still aligning with the isolation sources selected. Based on these analyses, two *Archaea* primer sets and two 16S bacteria primers sets were selected and validated.

Validation Study Sampling

An evaluation study was designed to confirm the presence/absence of selected markers (from the pyrosequencing data sets for *Archaea* and Bacteria) in biosolid samples and the presence/absence of these identified markers in animal waste samples (both raw animal manure and treated waste). However, a true method validation study according to the USEPA requirements was not possible due to the limited number of available biosolids and animal waste samples, the inability to collect samples representing different geographic areas and seasons of the year, and the inability to include samples on a masked ("blinded") basis to the analyst. Additionally, the feasibility of applying these biosolid markers was demonstrated in a field study analyzing surface waters proximal to sites subject to land application of biosolids.

Wastewater Treatment Plants. Five municipal wastewater treatment plants (WWTPs) in the Southeastern region of the United States contributed samples to help evaluate sensitivity of the candidate biosolid Microbial Source Tracking (MST) markers. The plants were chosen based on their different wastewater treatment processes (e.g., aerobic digestion, mesophilic anaerobic digestion, thermophilic

anaerobic digestion), thereby influencing the final biosolids product (e.g., Class A and Class B). All plants will remain anonymous for the purpose of this study.

Biosolid samples. Biosolid samples were collected from the five different WWTPs within the Southeastern region of the United States (Table 5). A minimum of two samples per participating plant was collected. The samples included dewatered "cake" and liquid biosolid products destined for land application. Samples 1, 3, 4, 6, 7, 8, and 9 are all classified as Class B and land-applied; samples 2, 5, and 10 are land-applied Class A products with two different treatment processes to produce its final product; and samples 6 and 7 are the liquid feed (treated) used to produce land-applied samples 1 and 3, respectively. Six samples were treated anaerobically, either under mesophilic or thermophilic conditions; four samples were treated aerobically, with no addition of heat; and one sample received lime stabilization, further reducing the microbial load (Table 5).

Treated animal samples. Swine, dairy, and poultry animal waste samples were collected in duplicate in May 2015. The treated swine and dairy effluent samples were collected from local swine and dairy lagoons that treat waste destined for land application. The poultry waste was a compost material that included poultry carcass and waste. Additionally, untreated animal waste from cows, chickens, and pigs were collected from a local farm. All animal waste samples were transported to the lab on ice. DNA was extracted in duplicate using the MoBio PowerSoil kit per the manufacturer's protocol, adjusting sample mass to 0.1g. All samples were stored at -20C until further molecular analysis.

Table 5.

Treatment Type, Biosolid Type and Form for All Biosolid Products Collected at Five WWTPs (A–E).

(It is important to note that same samples were products from the same plant but may be a different treatment or biosolid type.)

WWTP	Sample	Treatment Type	Biosolid Type	Biosolid Form	Dewatered (Yes or No)
A	1	Mesophilic, anaerobic	B	Cake	Yes
B	2	Thermophilic, anaerobic	A	Liquid slurry	No
C	3	Mesophilic, anaerobic	B	Cake	Yes
D	4	Aerobic	B	Liquid slurry	No
D	5	Aerobic, lime	A	Pellet	Yes
A	6	Mesophilic, anaerobic	B	Liquid slurry	No
C	7	Mesophilic, anaerobic	B	Liquid slurry	No
E	8	Aerobic	B	Liquid slurry	No
E	9	Aerobic	B	Cake	Yes
B	10	Thermophilic, anaerobic	A	Cake	Yes

Performance Evaluation Study Data Analysis

PCR assays targeting the four different biosolid markers were applied to determine presence/absence of these organisms in all biosolids and animal waste collected. Figure 4 illustrates the process used to validate markers. Conventional PCR assays were performed using a BioRad Thermocycler (BioRAd Thermocycler 96 well). The PCR product length was measured in comparison to a high molecular weight DNA 100bp ladder (Fisher BioReagents ™ exACTGene™ DNA Ladders) with agarose gel electrophoresis (2.5% in 1X TAE buffer) with Ethidium bromide staining. Gels were run for 120 minutes at 100V and interpreted using UV light in a BioRad Gel Doc ™ EZ Imager.

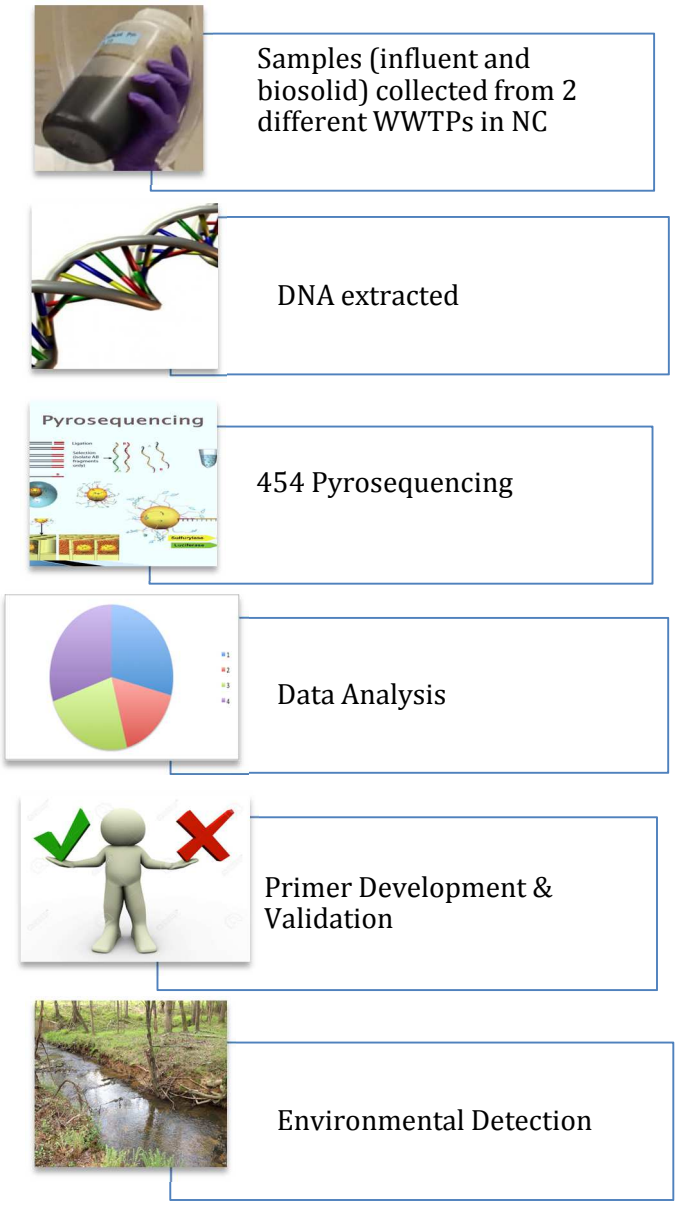

Figure 4. Process flow chart for developing biosolid MST marker.

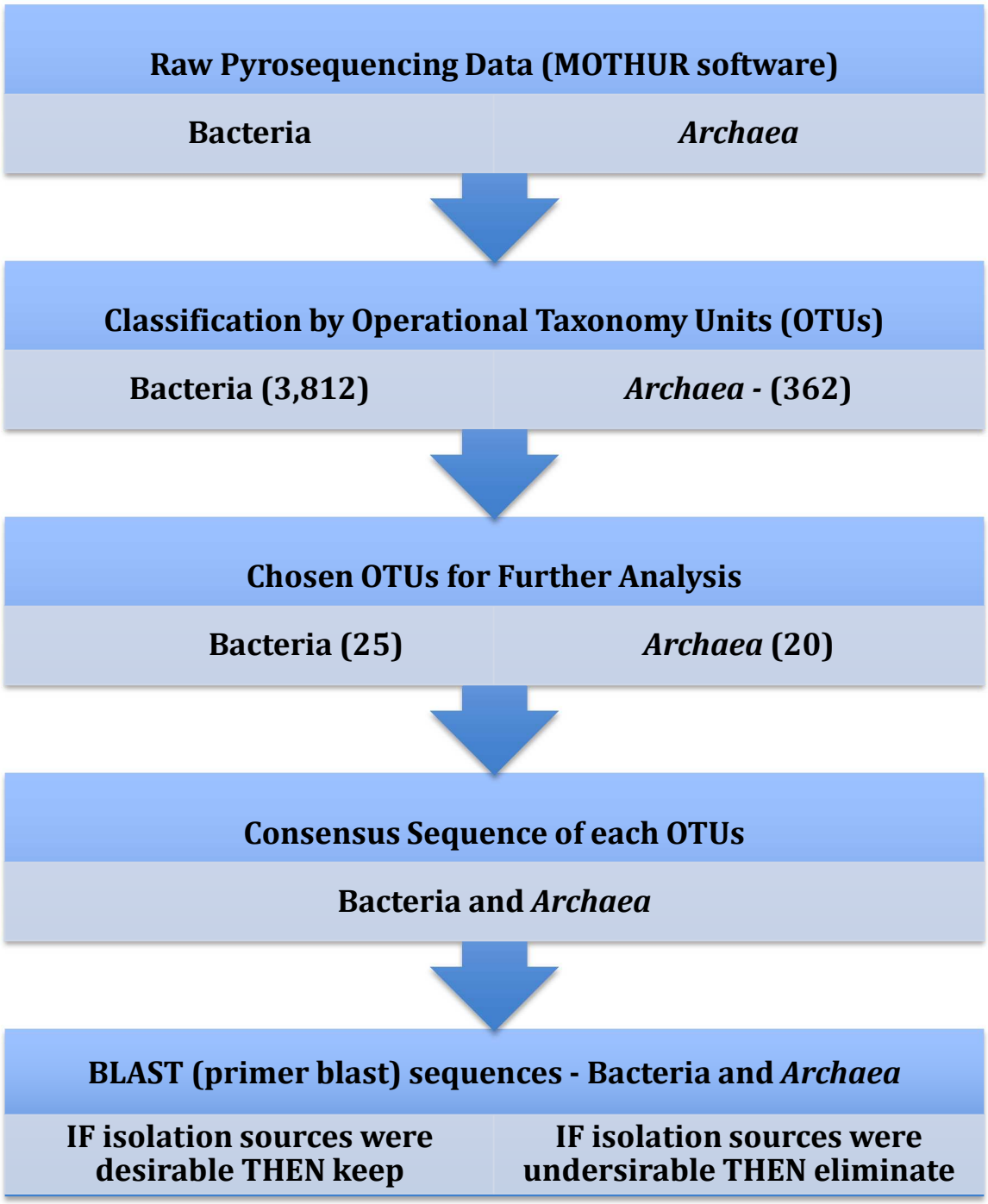

Figure 5. Process for analyzing Bacteria and *Archaea* pyrosequencing data.

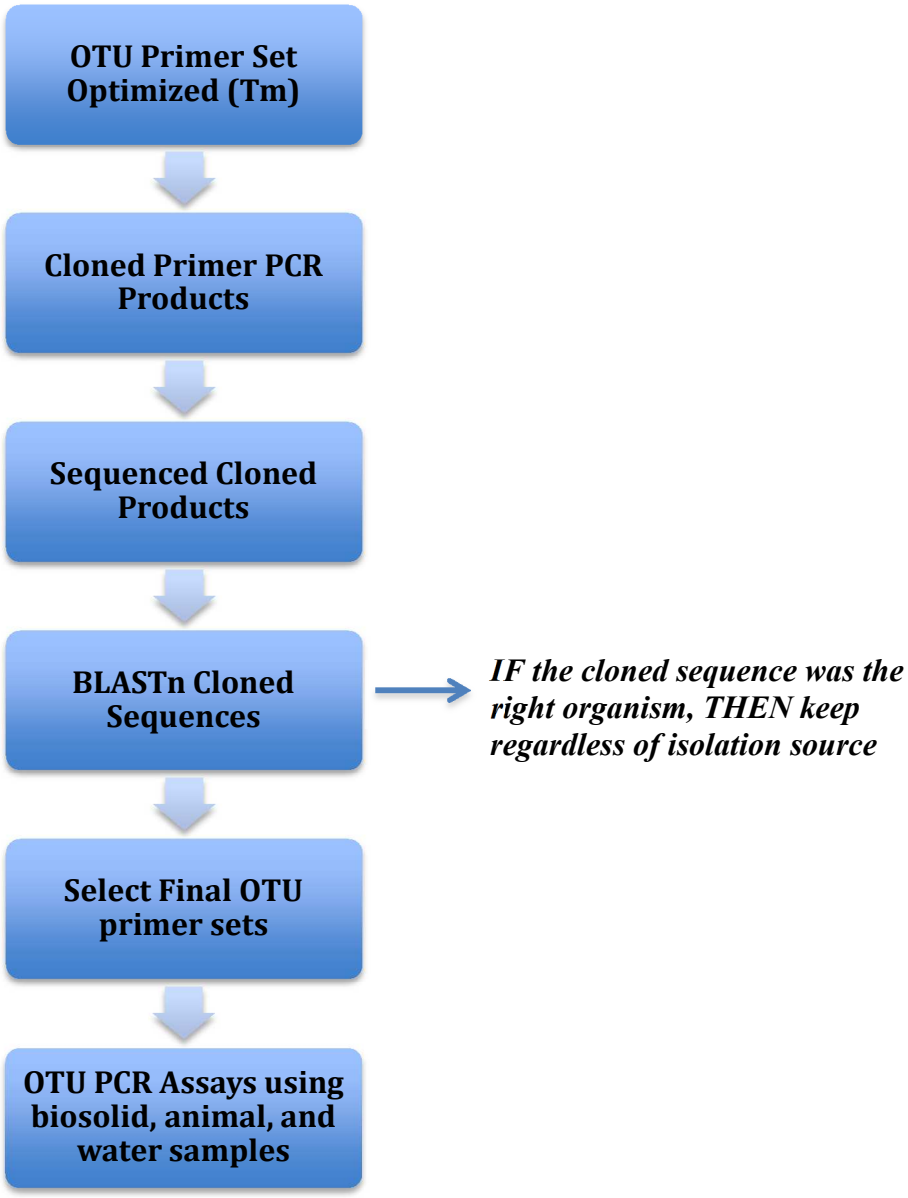

Figure 6. Process for designing primer sets for Bacteria and *Archaea* markers.

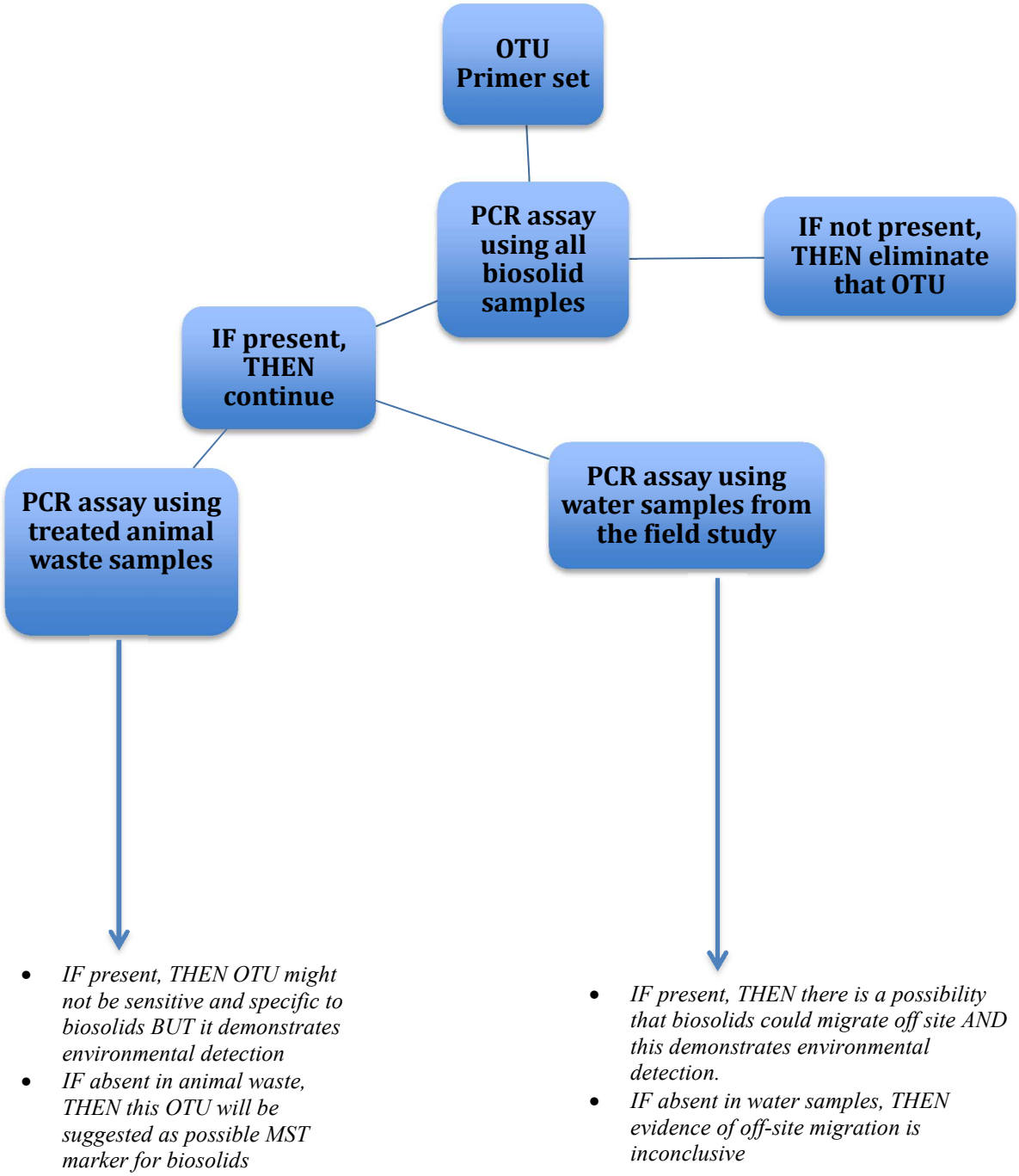

Figure 7. PCR assay procedure toward developing and evaluating biosolid MST markers.

Results

Pyrosequencing Sequences for Archaea and Bacteria Kingdoms

The results from the 454 data analysis yielded a total of 10,035 sequences belonging to the *Archaea* kingdom and approximately eight times as many for the bacteria community (80,304 sequences). Table 6 highlights the differences in the number of sequences for these kingdoms for influent and biosolid samples collected. At WWTP1, the number of sequences for the microbial community of the influent (point of entry in the WWTP1) samples collected for both *Archaea* and bacteria groups was lower than that after treatment and production of biosolids, which exits the WWTP for land application.

Table 6.

Total Pyrosequencing Sequences for *Archaea* and Bacteria Per Sample (Influent and Biosolids) Collected at WWTP1 and WWTP2

(WWTP) Sample	Number of *Archaea* seqs	Number of Bacteria seqs
WWTP1 – I1	396	13883
WWTP1 – I2	163	7703
WWTP1 – B1	2144	23213
WWTP1 – B2	6812	18629
WWTP2 – I1	270	2466
WWTP2 – I2	155	1562
WWTP2 – B1	52	8903
WWTP2 – B2	43	3945
Total	10035	80304

Taxonomy and targeted OTUs for Archaea and Bacteria

At the genus level of classification with 97% similarity, Table 7 describes the number of sequences assigned to the top 20 OTUs for *Archaea* (cut off of 0.01).

There were a total of 10,035 sequences classified in the *Archaea* kingdom across all samples collected (influent and biosolids) between both plants. The majority (66%) of the sequences for all *Archaea* identified a *Methanosarcina* as being classified in OTU001 (6,615 sequences). However, this OTU did not qualify as a suggested biosolids marker based on *in silico* testing (Table 8) because it was reported to be isolated from non-human sources. Additionally, *Methanobrevibacter* ARC_OTU004 (with 23 sequences, 0.23% of total *Archaea* population) also didn't qualify as a potential biosolid marker, despite being the only OTU that was shared across both plants, because it had been identified in pigs, lamas, humans, and buffalos. *Methanosaeta* ARC_OTU009 and unclassified-uncultured Archaea ARC_OTU016 (Tables 9 and 10, respectively) were the two OTUs that meet our *in silico* criteria as potentially specific to biosolids, leading to further lab analysis to determine the utility of these OTUs for primer development. ARC_OTU009 had 57 sequences all of which were found at WWTP1 and ARC_016 had 25 sequences and was also found only at WWTP1. The treatment process at this plant is anaerobic digestion, which would favor the enrichment of anaerobic microorganisms.

For Bacteria, at 97% similarity, 3,812 OTUs for the Bacteria kingdom were identified and classified at the genus level. Due to the large diversity in the bacterial community, a criterion to examine the OTUs that were shared across WWTPs for biosolid samples was used (Table 11). We used a command in the MOTHUR software that created a Venn diagram (Figure 8) of all of the OTUs shared across the two plants. We placed emphasis on the shared biosolids section because that is what is treated and destined for land application and it was our goal to develop a

universal marker. There were a total of 25 OTUs that were shared across both plants for all biosolid samples collected. OTUs 001, 030, and 040 had the most number of total sequences. *Leptotrichiaceae* 16S-OTU001, which in this case had the majority of the sequences (654), qualified as a suggested marker for biosolids based on *in silico* results (Table 13) and we were also able to identify an unclassified *Betaproteobacteria* BAC_OTU040 (Table 14) as a potential marker meeting our criteria prior to lab analysis; whereas, Table 12 illustrates an abundant OTU that did not qualify. In summary, *Archaeal* OTUs 009 and 016, and bacterial OTUs 001 and 040 were selected as candidate biosolid-specific markers. Marker HF183 was also selected for study based on its known specificity for human fecal waste.[14,67]

Conventional PCR for validation of suggested biosolid markers

Based on results from all biosolids collected from 5 different WWTPs (Table 15), we found the suggested biosolid markers in the majority of the samples tested. All five markers tested were found in all of the WWTPs that use anaerobic digestion to treat biosolids. For plants that use anaerobic treatment there were no differences in microbial detection when the operating temperature (mesophilic vs. thermophilic) was considered. However, the following differences were observed at WWTPs that treated biosolids aerobically: ARC_OTU016 was present in sample 4 (x2) from an aerobic digester but absent in samples 8 (x2) and 9 (x1), both from aerobic digesters. Biosolid markers and the HF183 marker were absent in sample 5 (x2), which received lime stabilization. There were no differences in dewatered vs. liquid form.

The suggested biosolid markers were tested using treated and untreated animal manure. Differences were observed for untreated animal manure (Table 16) vs. treated animal waste (Table 17). Samples collected from pigs, chickens, and cows were absent for HF183 and the Archaea biosolid markers (ARC_OTU009 and ARC_OTU016) but present for the bacterial biosolid markers (16S_OTU001 and 16S_OTU040). However, treated samples collected from dairy and swine lagoon samples were absent for HF183 but present for all biosolid markers. The composted poultry waste was absent for all markers tested.

Table 7.

Archaea Top 20 OTUs (Out of 362) and Number of Sequences Per OTU for Samples (Biosolids and Influent) Collected across WWTP1 and WWTP2

Each plant's biosolid sample is designated in the table by a "B" (B1 and B2 for the duplicate samples collected) and the same is true for influent samples ("I"). For the purpose of our study, we focused on biosolid samples only and the last two columns provide the total number of biosolid sequences for each OTU. (Note: cut off 0.01)

OTUs	WWTP1-B1	WWTP1-B2	WWTP1-I1	WWTP1-I2	WWTP2-B1	WWTP2-B2	WWTP2-I1	WWTP2-I2	WWTP1 Biosolid Total	WWTP2 Biosolid Total
1	1433	5182	8	0	0	0	0	0	6615	0
2	273	537	26	0	0	0	0	0	810	0
3	74	517	0	0	0	0	0	0	591	0
4	0	16	201	39	3	4	10	10	16	7
5	1	1	28	45	0	0	69	23	2	0
6	62	79	0	0	0	0	0	0	141	0
7	71	37	0	0	0	0	0	0	108	0
8	0	0	26	7	2	0	27	34	0	2
9	30	27	4	0	0	0	0	0	57	0
10	13	38	0	0	0	0	0	0	51	0
11	24	19	0	0	0	0	0	0	43	0
12	23	18	0	0	0	0	0	0	41	0
13	0	0	0	0	23	17	0	0	0	40
14	0	0	4	4	0	4	13	11	0	4
15	15	9	0	2	0	0	0	0	24	0
16	1	24	1	0	0	0	0	0	25	0
17	0	0	4	2	0	0	10	6	0	0
18	0	0	0	14	0	0	3	1	0	0
19	0	0	9	9	0	0	0	0	0	0
20	0	18	0	0	0	0	0	0	18	0

Table 8.

BLAST Results for Primer Pair Targeting the Consensus Sequence for *Archaea* OTU 001 at Genus Level for *Methanosarcina*

This primer pair was eliminated based on undesired isolation sources.

Primer Set (Product length: 216)			Primer (length, start, stop, Tm)	Sequence
			Forward (20, 5, 24, 59.96)	TACAATGCGGGAAACCGTGA
			Reverse (20, 220, 201, 59.97)	AACGGTTGAGCCGTCAGATT
GenBank #	**Organism**	**Title**	**Isolation Source**	
LN624324.1	uncultured archaeon	Microbial community dynamics during the mono fermentation of maize	mesophilic lab-scale biogas reactor	
KF419191.1	uncultured archaeon	Assessment of a biogas-generating microbial community in an industrial bioreactor	biogas plant	
AB973357.1	Methanosarcina thermophila TM-1	Methanosarcina thermophila sp. nov., a thermophilic, acetotrophic, methane-producing bacterium	diatomaceous shale formation	
		'Methanosarcina sp., a methanogenic archaeon isolated from a deep diatomaceous shale formation		
KF630672.1	uncultured archaeon	Microbial communities involved in biogas production from wheat straw as the sole substrate within a two-phase solid-state anaerobic digestion	digested from the up-flow anaerobic solid state reactor of a thermophilic (55degreeC) two phase two stage biogas reactor system biogas reactor system fed with wheat straw as sole substrate.''	
KF630671.1	uncultured archaeon	Microbial communities involved in biogas production from wheat straw as the sole substrate within a two-phase solid-state anaerobic digestion	biofilm on a carrier element taken from the anaerobic filter of a mesophilic (37C) two phase two stage biogas reactor system fed with wheat straw as sole substrate	
AB908268.1	uncultured archaeon	Inhibitory Effects of Ferrihydrite on a Thermophilic Methanogenic Community	thermophilic anaerobic digester	
KF971874.1	uncultured Methanosarcina sp.	Thermophilic anaerobic digestion of thermal-pretreated activated sludge correlation between microbial composition and process performance''	thermophilic sludge anaerobic digestion	
JX865673.1	uncultured archaeon	Archaea on human skin	human skin	
JX865665.1				
AB772284.1	uncultured euryarchaeote	Methane fermentation of stillage from Sweet potato-shochu making in a full-scale plant and energy recovery	a thermophilic anaerobic fixed bed reactor	

GenBank #	Organism	Title	Isolation Source
KC493221.1	uncultured archaeon	The analysis of microbial community in a thermophilic anaerobic digester running on swine manure by multiple approaches	swine manure: anaerobic thermophilic digester
KC203046.1	Methanosarcina thermophila	Isolation of methanogens from termite gut	gut - termite
AB721088.1	uncultured Methanosarcinales archaeon	Operation of a cylindrical bioelectrochemical reactor containing carbon fiber fabric for efficient methane fermentation from thickened sewage sludge	thickened sewage sludge of methane fermentor
HE805045.1	uncultured archaeon	Temperature increase from 55 degree C to 75 degree C in a two-phase biogas reactor results in fundamental alterations within the bacterial and archaeal community structure	anaerobic filter reactor of a thermophilic (55C) two phase two stage leach-bed biogas reactor supplied with rye silage in batches with a retention time of 21 days
NR_118372.1	Methanosarcina thermophila TM-1	PCR biases distort bacterial and archaeal community structure in pyrosequencing datasets	not identified
JF417891.1	uncultured archaeon	Characteristic microbial community of a dry thermophilic methanogenic digester: its long-term stability and change with feeding Microbial community in a thermophilic dry anaerobic cellulose digester and the change of community with feeding of garbage stillage discharged from an ethanol production process	dry anaerobic digester
AB541623.1	uncultured archaeon	Changes in archaeal community structure during the composting process of cattle manure	cattle manure compost
AB541661.1	uncultured archaeon	Archaeal community dynamics and detection of ammonia-oxidizing archaea during composting of cattle manure using culture-independent DNA analysis	cattle manure compost
FN994138.1	uncultured archaeon	Characterisation of microbial community structures in biogas fermentations with semi-continuous loading of different silages or manure as sole substrate at varying loading rates	long term biogas completely stirred tank reactor
HQ141839.1	uncultured Methanosarcina sp.	Prokaryotic diversity, composition structure, and phylogenetic analysis of microbial communities in leachate sediment ecosystems	leachate sediment

Table 9.

BLAST Results for Primer Pair Targeting the Consensus Sequence for *Archaea* OTU009 at Genus Level for *Methanosaeta*

This primer set was selected as possible biosolid marker based on isolation sources.

Primer Set (Product length: 73)			**Primer (length, start, stop, Tm)**	**Sequence**
			Forward (20, 178, 197, 59)	GGCCGGATAAGTCTCTTGGG
			Reverse (20, 250, 231, 59)	GCCTCGAGCCAGACAGTATC
GenBank #	**Organism**	**Title**	**Isolation Source**	
LN624353.1	uncultured archaeon	Determination of anaerobic degradation pathways through stable carbon isotope analysis in the two-stage anaerobic digestion of solid substrates	mesophilic UAF reactor	
LN624338.1	uncultured archaeon	Microbial community dynamics during the monofermentaion of maize respectively sugar beet silage	mesophilic lab-scale biogas reactor	
AB997116.1	uncultured archaeon	Characteristic functional microbial Communities and evaluate substrate effects on the methane fermentation performance in full scale and lab scale digesters	Sludge from full scale anaerobic digester	
KF551967.1	uncultured archaeon	Effects of the reduction of the hydraulic retention time and immobilization of microorganisms on anaerobic digestion and methanogenic community composition	lab-scale anaerobic digester	
HG967649.1	uncultured Methanosaetaceae archaeon	Microbial community composition and dynamics in high-temperature biogas reactors using industrial bioethanol waste as substrate	DNA sample from thermophilic biogas reactor	

Table 10.

BLAST Results for Primer Pair Targeting the Consensus Sequence for *Archaea* OTU016 at Genus Level for Unclassified Uncultured *Archaea.*

This primer set was selected as possible biosolid marker based on isolation sources.

Primer Set (Product length 167)			**Primer (length, start, stop, Tm)**	**Sequence**
			Forward (20, 53, 71, 59.76)	GCATGGGCTGTTCTTTGGTC
			Reverse (20, 218, 199, 59.97)	ACGGTTGAGTCGCAGGATTT
GenBank #	**Organism**	**Title**	**Isolation Source**	
LN624354.1	uncutured archaeon	Determination of anaerobic degradation pathways through stable carbon isotope analysis in the two-stage anaerobic digestion of solid substrates	mesophilic UAF reactor	
LN624331.1	uncutured archaeon	Microbial community dynamics during the monofermentaion of maize respectively sugar beet silage	mesophilic lab-scale biogas reactor	
AB997232.1	uncutured archaeon	Characteristic functional microbial Communities and evaluate substrate effects on the methane fermentation performance in full scale and lab scale digesters	Sludge from full scale anaerobic digester	
HG967648.1	uncultured methanogenic archaeon	Microbial community composition and dynamics in high-temperature biogas reactors using industrial bioethanol waste as substrate	DNA sample from thermophilic biogas reactor	
KP769486.1	uncultured archaeon	Unusually low TEX86 values in the transitional zone between Pearl River estuary and coastal South China Sea: Impact of changing archaeal community composition	transitional zone	
KM408628.1	uncultured archaeon	Impact of Methanosaeta harundinacea 6Ac and its quorum sensing on the performance and granulation of a UASB reactor fed with synthetic wastewater	anaerobic granules	
KM221256.1	uncultured archaeon	Linking microbial community structure to the S, Fe and N biogeochemical cycling in the hot springs at Tengchong geothermal fields, China	hot spring	
KJ476553.1	uncultured archaeon	Performance and microbial community profiles in an anaerobic reactor treated with simulated PTA wastewater: From mesophilic to thermophilic temperature	UAFB system treated with terephthalate contained wastewater	
KJ424795.1	archaeon enrichment culture clone	Microbial methane formation in deep aquifers of a coal-bearing sedimentary basin, Germany	coal-rich sediment	
KJ806548.1	uncultured archaeon	Investigation of the diversity of methanogenic archaea in 4 rural biogas digesters in Yunnan, China	anaerobic digester sludge	
KJ782207.1	uncultured euryarchaeote	Comparative studies on the archaeal diversity of two rural household biogas digesters in the temperate climate zones of Yunnan plateau	biogas digester sludge	
LC002078.1	uncultured archaeon	Presence of a novel methanogenic archaeal lineage in anaerobic digesters inferred from mcrA and 16S rRNA gene phylogenetic analyses	anaerobic granular sludge of UASB reactor treating sewage	

Figure 8. Bacterial Venn diagram illustrating the distribution of OTUs for biosolid samples between WWTP1 and WWTP2 (cutoff 0.03).

Table 11.

Bacterial OTUs Shared between WWTP1 and WWTP2 for Biosolid Samples Collected

(Note: cutoff 0.03)

	Number of sequences		
OTUs	**WWTP1**	**WWTP2**	**Total**
1	651	3	654
4	94	3	97
30	250	61	311
40	255	4	259
84	13	105	118
87	2	111	113
93	3	103	106
109	53	10	63
115	71	5	76
118	3	77	80
126	40	25	65
159	3	50	53
162	3	44	47
166	15	15	30
184	35	5	40
196	2	29	31
211	30	3	33
259	20	4	24
332	9	9	18
345	13	4	17
346	11	4	15
429	3	9	12
567	6	2	8
648	5	2	7
654	2	5	7

Table 12.

BLAST Results for Primer Pair Targeting the Consensus Sequence for Bacteria_OTU030 at Genus Level for Erysipelotrichaceae_incertae_sedis.

This primer set was eliminated based on undesired isolation sources.

Primer Set (Product length: 212)			**Primer (length, start, stop, Tm)**	**Sequence**
			Forward (20, 30, 49, 60.03)	AAAGGTATGGGCTCAACCCG
			Reverse (20, 241, 222, 60.11)	CGTTTACGGCGTGGACTACT
GenBank #	**Organism**	**Title**	**Isolation Source**	
KF843495.1	uncultured bacterium	Metagenomic study of the fecal microbiota in a southern Indian rural population	Homo sapiens: pooled stool from elderly group	
JX851672.1	uncultured bacterium	Changes in gut flora of diabetic and non-diabetic Indian individuals	Homo sapiens: feces	
KF230260.1	uncultured bacterium	Direct Submission	Homo sapiens: stool sample	
JN567803.1	uncultured Firmicutes bacterium	Molecular Analysis of Bacterial and Viral Bioaerosols in Concentrated Animal Feeding Operations	CAFO bioaerosol collected from concentrated animal feeding operations	
JQ186851.1	uncultured bacterium	Distinct distal gut microbiome diversity and composition in healthy children from Bangladesh and the United States	human stool	

Table 13.

BLAST Results for Primer Pair Targeting the Consensus Sequence for Bacteria_OTU001 at Genus Level for Leptotrichiaceae

This primer set was selected as possible biosolid marker based on isolation sources.

Primer Set (Product length: 111)			**Primer (length, start, stop, Tm)**	Sequence
			Forward (20, 89, 108, 60.11)	GGTGGACGGAACTACACGAG
			Reverse (20, 199, 180, 60.04)	CCCCTAGCTTTCGCACTTCA
GenBank #	**Organism**	**Title**	**Isolation Source**	
AB902786.1	uncultured bacterium	Recovery and biological oxidation of dissolved methane from UASB-treated sewage effluents using post-treatment with two-stage closed down-flow hanging sponge system	Down-flow Hanging Sponge (DHS) reactor treating sewage after Up-flow Anaerobic Sludge Bed (UASB) reactor	
JX040374.1	uncultured bacterium	Direct Submission	waste water	
HM773500.1	uncultured bacterium	16S rDNA sequences of biofilm-consortium on RABC of food wastewater treatment plant	biofilm-consortium on RABC of food-wastewater treatment plant	
AB948029.1	uncultured prokaryote	Effects of anaerobic protozoa on treatment efficiency and microbial community structures in uasb reactors fed with domestic sewage	anaerobic UASB reactor fed with domestic sewage	
JF342096.1	uncultured bacterium	Molecular survey of concrete sewer biofilm microbial communities	concrete sewer biofilm	
GU044518.1	uncultured bacterium	Bacterial diversity in rhizosphere soil from Antarctic vascular plants of Admiralty Bay, maritime Antarctica	soil from Brazilian Antarctic Station	

Table 14.

BLAST Results for Primer Pair Targeting the Consensus Sequence for 16S Bacteria_OTU040 at Genus Level for Betaproteobacteria

This primer set was selected as possible biosolid marker based on isolation sources.

Primer Set (Product length: 201)			Primer (length, start, stop, Tm)	Sequence
			Forward (20, 44, 63, 59.89)	AACCTGGGAACTGCGATTGT
			Reverse (20, 244, 225, 60.11)	CATCGTTTAGGGCGTGGACT
GenBank #	**Organism**	**Title**	**Isolation Source**	
KF956489.1	uncultured Rhodocyclaceae bacterium	Bioreduction of vanadium (V) in groundwater by autohydrogentrophic bacteria: mechanisms and microorganisms	batch anaerobic bioreduction vanadate in contaminated groundwater	
KF830609.1	uncultured bacterium	Effect of intermittent aeration cycle on nutrient removal and microbial community in a fluidized bed reactor-membrane bioreactor combo system	domestic wastewater	
KC551571.1	uncultured bacterium	The bacterial community of activated sludge from 4 wastewater treatment plants	activated sludge	
AB516070.1	uncultured bacterium	polyhydroxyalkanoates (PHA)-accumulating organisms in full-scale wastewater treatment plants	activated sludge	
AB529670.1	bacterium YO-16	Microbial Community Analysis in the Roots of Aquatic Plants and Isolation of Novel Microbes Including an Organism of the Candidate Phylum OP10	rhizoplane of reed	
GU179649.1	uncultured beta proteobacterium	Microbial diversity profiles of fluids from low-temperature petroleum reservoirs with and without exogenous water perturbation	oil well	
HM773450.1	uncultured bacterium	16S rDNA sequences of biofilm-consortium on RABC of food-wastewater treatment plant	biofilm-consortium on RABC of food-wastewater treatment plant	
GU917918.1	uncultured bacterium	Microbial diversity analysis using pyrosequencing of small-subunit ribosomal RNA without PCR amplification	activated sludge	
EU834779.1	uncultured bacterium	Comparison of methods for the extraction of nucleic acid from activated sludge	lab scale EBPR-activated sludge	
AB504619.1	uncultured bacterium	Biological oxidation of dissolved methane in effluents from anaerobic reactors using a down-flow hanging sponge reactor	methane oxidizing DHS reactor	
GQ871697.1	uncultured bacterium	Effect of aspartate and glutamate on the fate of enhanced biological phosphorus removal process and microbial community structure	sequencing batch reactor (SBR) operated for enhanced biological phosphorus removal (EBPR)	
GQ455779.1	uncultured bacterium	Population dynamics in a sequencing batch reactor fed with glucose and operated for enhanced biological phosphorus removal	sludge from sequencing batch reactor (SBR) operated for enhanced biological phosphorus removal (EBPR)	

GenBank #	Organism	Title	Isolation Source
AB479732.1	uncultured bacterium	Characterization of the microbial community in the anaerobic/oxic/anoxic process combined with sludge ozonation and phosphorus adsorption	activated sludge
EU104186.1	uncultured bacterium	Evidence for the Comamonadaceae as determinants of activated sludge settling performance	activated sludge
AB280287.1	uncultured bacterium	Identification of the bacterial community involved in methane-dependent denitrification in activated sludge using DNA stable-isotope probing	Methane-consuming sludge
DQ413110.1	uncultured bacterium	Comparative Analysis of Microbial Communities from Culture-dependent and -independent Approaches in an Anaerobic/Aerobic SBR Reactor	EBPR sludge
AB205805.1	uncultured bacterium	Identification of Acetate- or Methanol-Assimilating Bacteria under Nitrate-Reducing Conditions by Stable-Isotope Probing	denitrifying activated sludge
KF981612.1	uncultured Rhodocyclaceae bacterium	Systematic analysis of performance and microbial community of an activated sludge fed with ozonated sludge at different ozone dose for sludge reduction	activated sludge
HM590828.1	Azonexus sp. HME6654	Direct Submission	pond
FM200936.1	uncultured bacterium	Diverse and distinct bacterial communities induced biofilm fouling in membrane bioreactors operated under different conditions	laboratory-scale membrane bioreactors
EU542357.1	uncultured bacterium	Attached bacterial populations shared by four species of aquatic angiosperms	pre-washed leaves of Vallisneria americana (wild celery) from Chesapeake Bay
KC189690.1	uncultured bacterium	Differences in Bacterial Community Structure on Hydrilla verticillata and Vallisneria americana in a Freshwater Spring	Wakulla Spring
AB292421.1	uncultured bacterium	Microbial community analysis of PHA accumulating organisms in full-scale wastewater treatment plants by buoyant density separation and fluorescence in-situ hybridization	PHA-accumulating organisms in full-scale wastewater treatment plants
JN125670.1	uncultured beta proteobacterium	Microbial biofilm-community structure dynamics during bioreduction of nitrate, sulfate and para-chloronitrobenzene in a hydrogen-based membrane biofilm reactor	biofilm sample in membrane biofilm reactor that bio reduced oxidized contaminants in drinking water

Table 15.

Biosolid Samples Collected from Five Different WWTPs

Conventional PCR results revealed presence/absence of suggested biosolid microbial markers and HF183 human specific marker.

WWTP	Biosolid Type	Treatment	Sample Month	Sample #	Suggested Biosolid Markers				HF183
					OTU 001	OTU 009	OTU 016	OTU 040	
A	Class B	Mesophilic Anaerobic	January	1a	P	P	P	P	P
			March	1b	P	P	P	P	P
B	Class A	Thermophilic, anaerobic	January	2a	P	P	P	P	P
			March	2b	P	P	P	P	P
C	Class B	Mesophilic, anaerobic	January	3a	P	P	P	P	P
			March	3b	P	P	P	P	P
D	Class B	Aerobic	January	4a	P	P	P	P	P
			March	4b	P	P	P	P	P
D	Class A	Aerobic, lime	January	5a	A	A	A	A	A
			March	5b	A	A	A	A	A
A	Class B	Mesophilic, anaerobic	January	6a	P	P	P	P	P
			March	6b	P	P	P	P	P
C	Class B	Mesophilic, anaerobic	January	7a	P	P	P	P	P
			March	7b	P	P	P	P	P
E	Class B	Aerobic	January	8a	P	P	A	P	P
			March	8b	P	P	A	P	P
E	Class B	Aerobic	March	9a	P	P	P	P	P
			April	9b	P	P	A	P	P
B	Class A	Thermophilic, anaerobic	April	10a	P	P	P	P	P
			April	10b	P	P	P	P	P

P Presence of marker A Absence of marker

Table 16.

Untreated Animal Waste Samples (Pig, Chicken, Cow) Collected from a Local Farm and Tested for Presence/Absence of Biosolid Microbial Markers

	Untreated Animal Manure				
	Suggested Biosolids Markers				
Samples	OTU 001	OTU 009	OTU 016	OTU 040	HF183
Pig	P	A	A	P	A
Chicken	P	A	A	P	A
Cow	P	A	A	P	A

P Presence of marker A Absence of marker

Table 17.

Treated Animal Wastes Samples (Dairy Lagoon, Swine Lagoon, and Poultry Compost) Tested for Presence/Absence of Biosolid Microbial Markers and HF183 Human-Specific Marker

	Treated Animal Manure				
	Suggested Biosolids Markers				HF183
Samples	OTU 001	OTU 009	OTU 016	OTU 040	
Dairy - 1	P	P	P	P	A
Dairy - 2	P	P	P	P	A
Swine - 1	P	P	P	P	A
Swine - 2	P	P	P	P	A
Poultry – 1	A	A	A	A	A
Poultry - 2	A	A	A	A	A

P Presence of marker A Absence of marker

Discussion

The purpose of this study was to identify novel microbial source tracking (MST) markers that were sensitive and specific to biosolid materials. In the event of successful marker identification, the intent is to use these markers in future field studies where biosolids are land-applied, evaluating the impact of this practice on water quality. To achieve our objective, high-throughput pyrosequencing was used to sequence the microbial community of two different types of biosolids at two different WWTPs. We identified OTUs in the Bacteria and Archaea kingdoms for each type of biosolid sampled (Class A and Class B). Further *in silico* analysis generated four primer sets that targeted specific microbial sequences of interest from the Bacteria and *Archaea* kingdoms, namely an unclassified *Betaproteobacteria, Leptotrichiaceae, Methanosaeta*, and an unclassified, uncultured *Archaea*.

We conducted laboratory tests to evaluate these primer sets for their presence/absence in biosolids and animal waste. Evaluation results revealed sensitivity for biosolids—all microorganisms of interest were present in biosolid samples collected across five different WWTPs in the Southeastern region of the United States. All plants that used anaerobic digestion resulted in detection of all the biosolid markers and the HF183 marker. However, other observations suggest that the sludge treatment method is a factor in detection of the markers. Aerobically treated samples had differences in the presence/absence of the targeted microorganism. Sample 4 and Sample 5 were both mesophilic aerobic digested products but sample 5 received an additional lime stabilization treatment, which

resulted in the absence of all markers. The addition of lime increases the pH and kills the microorganisms present. Sample 8 and sample 9 were both obtained from plants using aerobic digestion. We observed the absence of OTU016 in sample 8 (x2) and the same OTU was absent in one of the replicates from sample 9.

The observed differences for presence/absence of targeted markers in untreated and treated animal manure were similar to biosolids because treatment made a difference in the microorganisms detected. We detected the presence of all of the OTUs in the treated dairy and swine waste (obtained from anaerobic lagoons). However, we did not detect any markers in the composted poultry waste. Because composting is a high-temperature process, it is possible that very different microorganisms would be selected compared to anaerobic digestion.

Based on our findings, HF183 could help differentiate biosolid contaminants because it was not detected in untreated and treated animal waste. HF183 is a human-specific marker that has been used to track human fecal pollution because it is capable of distinguishing human fecal waste from other animal waste products.[14,67] However, we would expect to also find HF 183 in other samples originating from human waste, including septic tanks that are often present in the agricultural settings where biosolids are land applied. HF183 and a biosolid marker, specific to highly treated waste, could be combined to address the specificity issue we observed with our biosolid markers—HF183 was biosolid-specific and our biosolid markers were biosolid-sensitive. However, one concern to consider with using HF183 is the proximity of communities and houses to the creeks that could

have leaking septic tanks, which would contribute to the fecal pollution and possibly the detection of HF183 in water samples tested.

Sensitivity and specificity were important parameters to consider in developing novel markers for MST. Sensitivity is defined as the ability to detect the presence of that targeted source when the source is present, whereas the specificity is the ability to not detect the targeted source when the source is not present. Previous studies have yet to identify a perfectly (100%) sensitive and specific marker for any host source. A recent study conducted by Johnston et al. (2013) assessed the use of two human-specific markers (*Bacteroidales* HF183 and *Methanobrevibacter nif*H) to identify human fecal contamination. The authors concluded that no bacterial marker is completely host-specific[14,19,67] but that a combination of markers can increase the probability of correctly identifying a host source. Similarly, we found our biosolid markers in the majority of the biosolid samples collected but showing evidence of cross-reactivity in treated animal samples, indicating that the primers were biosolids-sensitive but not biosolid-specific. To increase the degree of confidence in a suggested marker, it is recommended to use more than one marker in combination.[67,68]

Several studies have examined and characterized the microbial diversity of samples from wastewater treatment plants at various stages throughout the treatment process, demonstrating the presence of a wide range of microorganisms.[15,16,19-21,68-72] In some studies, researchers were able to identify common core groups of microorganisms across different geographical locations.[15,16,21,71] This pilot study was designed to determine if we could detect the

presence of novel markers in biosolids and our results demonstrate, on a smaller spatial and temporal scale, the presence of common microorganisms in five WWTPs producing various types of biosolids.

Evidence supporting our hypothesis that source-specific markers could be identified in this complex wastewater treatment infrastructure has been documented in studies analyzing WWTP influent where researchers developed *Lachnopiraceae* markers to track sewage spills in water sources.[19,71,73,74] Differences in the presence/absence of OTU009 and OTU016 were observed in untreated animal waste and treated animal waste. These markers were absent in untreated animal waste but present in treated animal waste. These findings suggest that treatment matters and it enriches and/or reduces certain types of microorganisms; therefore, it appears that similar anaerobic processes to treat waste (regardless of the source) can select for the targeted organisms represented by OTU009 and OTU016. Efforts to develop novel biosolid source tracking markers have been limited; however, findings indicate that *Chloroflexi's* presence in biosolids and absence in soils exhibits promise for the microorganism's use as a potential biosolids source-tracking marker.[50,51]

Studies analyzing influent or effluent wastewater typically used the most abundant OTU for microbial marker development,[20,68,71,74] however we selected more specific OTUs that had isolation sources most similar to biosolids or the digestion process identified *in silico*. Since the objective was to develop a biosolids microbial source-tracking marker, this approach helped eliminate microorganisms that could potentially cross-react with other environmental sources of pollution.

Selecting less prevalent OTUs did not present an issue regarding the level of sensitivity when detecting these sequences in biosolids. Using PCR, we successfully detected and amplified the low abundant but more specific sequences in our environmental samples. We used these sequences to develop markers that were more specific to biosolids based on isolation sources identified *in silico*.

Additionally, two of the sampled biosolids sets (8 and 9) were negative for the anaerobic *Archaea* markers (ARC_OTU009 and ARC_OTU016), likely due to the non-thermal, aerobic treatment process used at the plant of origin. Because most Archaea are found in anaerobic environments, it is not surprising that the archaeal markers were absent from aerobically digested biosolids.

Literature indicates that most traditional fecal indicator measures fail in identifying specific sources of pollution because they are not source-specific; therefore, they fail to identify causes of water contamination.[75,76] MST is essential in identifying and distinguishing sources of pollution to help estimate health risks and to help identify effective strategies for remediation of water contamination. This is a growing field and additional research is needed to better characterize the microbial community of land-applied waste and to develop novel MST markers to aid in identifying and remediating sources of pollution. The main goal of this novel exploratory pilot study was to develop biosolid markers capable of detection in various biosolid samples. Combining PCR and pyrosequencing allowed us to identify target microorganisms that were low in abundance and not typically identified in environmental samples. We were able to detect the biosolid markers in other biosolid samples within our region. Unfortunately, we were unable to sample

biosolids from diverse geographical locations, therefore, we attempted to address this sampling limitation by selecting five diverse wastewater treatment plants representing four treatment processes producing both Class A and Class B biosolids. We cannot conclude the utility of these suggested biosolid microbial markers without further research that would involve a large sampling study collecting various treated animal waste and biosolids, statistically testing the effectiveness of these markers as potential MST candidates.

Despite our study being exploratory and small in scale, the results have implications that are impactful and strengthened by the call for biosolid source-tracking markers from regulatory agencies such as United States Geological Survey (USGS) [77] and local WWTP municipalities. In effectively assessing water quality and remediating polluted water sources, distinguishing sources of pollution is imperative and this research is a first step in developing biosolid-sensitive and -specific source tracking markers. These efforts could provide useful tools for the USEPA and WWTPs to better monitor and manage land application disposal practices to better protect the environment and to prevent human exposures.

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CHAPTER 4. MICROBIAL SOURCE TRACKING TO EVALUATE MICROORGANISMS FOLLOWING LAND APPLICATION OF BIOSOLIDS TO NEARBY WATER SOURCES IN NORTH CAROLINA

Summary

Land application is the primary means of managing and disposing of biosolids, the solids removed during treatment of municipal wastewater. Following land application, surface waters may become contaminated with biosolid materials through overland flow or groundwater transport, but transport is difficult to measure using traditional indicators that are not specific to biosolids. For this study we used microbial source tracking (MST) techniques to demonstrate environmental detection of four candidate biosolid microbial markers and a human-specific marker HF183 at a land application field site, as compared to a reference field site. Conventional PCR was used to evaluate water samples (n=34) collected upstream and downstream from a creek that flows through a field subjected to land application of biosolids, as well as from a reference creek nearby that is not exposed to land application of biosolids. Three of the four candidate markers were consistently detected in the upstream, downstream, and reference sites. However, the fourth marker (OTU016, targeting an unclassified, uncultured *Archaea*) was detected only in the water samples collected from the creek exposed to land application following a heavy rain event. These two positive water samples, collected both upstream and downstream, were also positive for HF183, the most sensitive and specific marker for identifying human-specific fecal contamination. Hence, we propose that a combination of

OTU016 and HF183 as MST markers may be helpful in detecting the presence and possibly tracking the transport of biosolids removed during municipal treatment of wastewater and disposed through land application into the environment. To the best of our knowledge this is the first study to attempt to use MST biomarkers to detect the occurrence of biosolids from land application in nearby surface waters. The implications from these findings have the potential to inform future approaches for detecting the occurrence and tracking the fate and transport of biosolids into surface waters and ultimately to guide surveillance policies to monitor the public health impact of wastewater treatment and disposal.

Introduction

Land application is the primary means of managing and disposing of biosolids. Approximately 60% of the 5.6 million dry tons of sewage sludge disposed annually in the United States is land-applied.[2,78] In accordance with regulations and guidelines established by the U.S. Environmental Protection Agency (USEPA), Class A and Class B biosolids can be applied on fields to ensure the safety of the public and the environment. These regulations and guidelines are encoded in Chapter 40, Part 503 of the Code of Federal Regulations (40 CFR Part 503).[1] However, the practice of land application may contribute to the introduction of pathogens and other contaminants in the environment.[79] Depending on the condition of the sites (e.g., climate, soil moisture, soil texture) and the proximity to surface waters, off-site transport may occur during rain events, impacting surface waters with the potential for exposure to nearby communities.

Following land application, surface waters may serve as potential routes of pathogen exposure because they may serve as a source of drinking water, fishing, or recreation for downstream communities. Surface waters as well as ground waters risk contamination due to run-off during rain events.[39,41,54-56] A limited number of studies have investigated transport of microorganisms follow land application with conflicting results. One study reported detection of *C. perfringens* and *E. coli* in groundwater samples post-application at depths of 1.2m and 2.0m.[80] Using *E.coli* genetically fingerprinted to be associated with biosolids, another study demonstrated off-site transport of *E. coli* following a rain event.[45] Indeed, *E. coli, Enterococcus*, and *C. perfringens* have all been detected after land application and during rain events.[47] However, one 8–10 year study that examined water and soil for fecal coliforms and fecal streptococci reported no impact.[81] Tracking biosolids transport and detecting its occurrence in environmental samples would help determine whether land application as currently practiced is impacting water and environmental quality. To further investigate biosolids run-off, it is important to be able to identify the origins of pollution. Non-point source pollution is a cumulative effect; without specific indicators (i.e., human-specific), non-point source pollution presents a challenge in trying to inform WWTP management about best practices. Development of microbial-source tracking (MST) markers to distinguish biosolids from other sources of pollution would effectively identify the origin of pollution to assess environmental sustainability of this practice and to help identify effective strategies for remediation of water contamination.

Microbial source tracking is an approach used to track microorganisms in the environment and is a growing field of study that is still under development; however, it has been instrumental in identifying sources of fecal contamination.[76] Studies have concluded that microbial markers such as anaerobic intestinal bacteria *Methonobrevibacter smithii* and *Bacteriodales,* based on human specificity, are capable of confirming the presence of human waste.[10-14] In a field study tracking bioaerosols released during high wind conditions following the land application of biosolids, MST methods led to successful detection of microorganisms (e.g., *Chloroflexi*, *Euryarchaeota*, and *Clostridium bifermentans*) found in biosolids. The study concluded that windy conditions factor in off-site transport of biosolid contaminants via bioaerosols. [51]

The objective of this research was to demonstrate environmental occurrence of novel biosolid microbial markers at a land application field site. To the best of our knowledge no study has used MST to detect the occurrence of novel biosolid markers in surface waters. This research is novel and has the potential to provide tools to wastewater treatment utilities and the USEPA for best biosolids management practices.

Materials and Methods

Field Study Site

To conduct this field study, we identified two creeks (Creek A and Creek B) to collect water samples. Creek A served as a reference site where reportedly no biosolids from the participating plant had been applied on adjacent fields. We are unaware if other plants land-applied biosolids upstream of these fields. Fields nearby

Creek B received land-applied biosolids and samples were collected upstream and downstream of these fields.

Water sampling

An initial sampling occurred in April 2014 and samples were then collected weekly, every Tuesday morning between 8:30am to 10:30am unless a rain event occurred. We also obtained samples following one heavy rain event on May 16, 2014, from sites at both Creek A and Creek B. At each location (e.g., Upstream, Downstream, Reference), 5 liters (L) of surface water were collected. Because the reference site was located underneath a bridge, we used a water-sampling bucket (designed in-house) to collect the samples. To obtain samples from upstream and downstream sites, we inserted a Nalgene bottle into the creek at a designated area where flow was consistent with attempts made not to disturb the sediments and to sample upstream of the collector. All bottles were capped and immediately placed in ice-packed coolers for transport back to the lab for analysis. Any material that settled was re-suspended before analysis.

Water sample analysis

a. IDEXX Quanti-Tray Colilert and Enterolert. Using the Colilert and Enterolert USEPA-approved methods that are included in Standard Methods for Examination of Water and Wastewater,[84] the concentrations of *Escherichia coli*, fecal coliforms, and enterococcus were analyzed in the water samples. The most probable number (MPN) was calculated using an MPN calculator.

b. Coliphage testing. Method 1602 was used to detect male-specific (F+) and somatic coliphages in water by the single agar layer (SAL) procedure.[82] After

overnight incubation, phage-induced lysis zones (plaques) were counted and totaled for all plates from a single sample. The quantity of coliphage in a sample was expressed as plaque forming units (PFU)/100 mL.[82]

c. Clostridium perfringens. The most probable number assay using iron milk media was used to test for *Clostridium perfringens*. The media was prepared by aseptically adding a can of fat-free evaporated milk to a 500 mL graduated cylinder. A 2% ferrous sulfate solution was prepared using 60mL of sterile DI and 1.2 g of ferrous sulfate, and 50 mL was added to the evaporated milk. The volume was brought to 500 mL by adding sterile DI water. After the milk was mixed with the ferrous sulfate solution, volumes of 10mL were dispensed into 15 sterile tubes (per sample) and 10 ml of sample were added to each tube. The tubes were incubated at 42°C for 18–24 hrs and then observed for stormy fermentation indicative of *Clostridium perfringens*.

d. Molecular analysis of water samples for source tracking markers. 200 mL of water samples were filtered using a 0.22 µm Durapore® (Millipore, Billerica, MA) membrane. All water samples were filtered in triplicate and the filter membrane was immediately placed in a cryogenic tube and stored at -80°C until molecular analysis was conducted. At such time, filters were transferred to PowerWater tubes for DNA extraction, following the protocol provided in the MoBio PowerWater kit (MoBio, Carlsbad, CA, and Thermo Scientific, Wilmington, DE). The extracted DNA was used for detection of *Bacteriodales* HF183 and novel biosolids markers targeting sequences in *Betaproteobacteria, Leptotrichiaceae, Methanosaeta*, and an unclassified uncultured *Archaea*. HF183 is a known human-specific indicator used to

distinguish human fecal pollution from other sources of pollution. It was used in this study as a confirmatory marker to help distinguish human pollution from animals, which were known to graze field sites. For each DNA target, PCR was carried out in reaction volumes of 25 µL using a BioRad CFX96™ Real-Time System (C1000 Touch™ Thermal Cycler). All PCR products were analyzed in duplicate by electrophoresis at the Lineberger Comprehensive Cancer Center (LCCC) Genomics Core service center at the University of North Carolina using an Agilent 2200 Tapestation.

e. Data analysis. A Fisher's Exact test was performed comparing the occurrence and concentrations of the different indicators tested at the different sites. An ANOVA test was performed to test the differences in mean concentrations of fecal indicator bacteria by location. For any indicator where a difference was found, we performed Tukey-Kramer test to determine the pairwise differences ($P<0.05$).

Results

Fecal Indicators in Surface Waters Collected

All three sites sometimes exceeded the North Carolina state standards for ambient freshwater water quality (Table 19). Using an *E. coli* standard for statistical threshold value (STV) of 235 CFU/100mL, 46% (n=6) of the downstream samples exceeded the regulatory threshold while 13% (n=1) of the upstream samples and 15% (n=2) of the reference stream samples were out of compliance. These proportions were different using an Enterococcus standard. Using an Enterococcus STV of 61 CFU/100mL, 70% (n=9) of the downstream samples exceeded the

threshold while only 63% (n=5) of the upstream samples were out of compliance and, 46% (n=6) of the reference samples were out of compliance (Table 20).

Alternative indicators including F+ coliphages, somatic coliphages, and Clostridia were also detected in these water samples. F+ coliphages, an indicator for enteric viruses, were detected in 31% (n=13) of the downstream samples at an average (SD) Log10 transformed concentration of 0.18 (0.32) PFU/100mL. However, the average concentrations were actually higher in the upstream (0.36 ± 0.58 MPN/100mL) and reference sites (0.54 ±0.72 MPN/100mL). A similar pattern was not observed for somatic coliphages or for clostridia. For both of these indicators, the downstream site did have the highest average concentrations, followed by the reference then the upstream sites (Table 21).

Water samples collected from the upstream, downstream, and reference sites consistently tested positive for fecal indicators (Figures 9–11). The downstream site had the highest concentration of fecal indicator bacteria for all indicators except F+ coliphages (Table 21). Whereas concentrations of each indicator were lower for the upstream and reference sites, typically they were within an order of magnitude of the concentrations measured at the downstream site. Also, we observed an increased concentration of fecal indicators after the May 16, 2014, rain event for all three sites and all three-indicator bacteria (Figures 9–11). These concentrations returned to baseline by the following time point. Differences in mean concentrations of fecal indicator bacteria by location were found for only somatic coliphages where the mean (SD) of Log10 transformed concentrations were 0.84 (1.03), 2.11 (0.64), and 1.52 (0.77) for the upstream, downstream, and reference sites, respectively

(p=0.005). Additional tests of the differences found for somatic coliphages determined that the upstream mean concentration differed from the downstream concentration (p=0.003).

Microbial Source Tracking Markers Tested in Water Samples

Pyrosequencing helped identify four sequences (targeting *Betaproteobacteria, Leptotrichiaceae, Methanosaeta*, and an unclassified uncultured *Archaea* designated OTU040, OTU001, OTU009, and OTU016, respectively) suggested as candidate biosolids microbial markers based on their presence in biosolids materials (Chapter 3). Based on the presence in biosolids from the previous analysis, all water samples collected from creeks adjacent to land-applied fields were tested for presence/absence of these biosolids markers and the HF183 marker (Table 18); there were a total of 34 water samples collected. Upstream samples could not be collected after sample 22, on May 27, 2015, because the creek was dry. Water sample results suggested the presence of all biosolid markers with the exception of OTU016, which was present only in two samples (upstream sample 16 and downstream sample 17). Because three of our markers appeared to be ubiquitous, our findings suggest a lack of specific association of these markers with samples from sites where biosolids were applied.

Table 18. Surface Water Samples Collected from a Creek Adjacent to Biosolid Land Application Sites (Upstream and Downstream) and a Creek (Reference) That is Nearby but Not Subject to Land Application of Biosolids

Conventional PCR was used to determine presence/absence of biosolid microbial markers and the human specific marker HF183.

Sample Date	Sample	Location	Biosolid Microbial Markers				HF183
			ARC_OTU009	ARC_OTU016	16S_OTU001	16S_OTU040	
4.22.14	1	upstream	P	A	P	P	A
	2	downstream	P	A	P	P	A
	3	reference	P	A	P	P	P
4.29.14	4	upstream	P	A	P	P	A
	5	downstream	P	A	P	P	A
	6	reference	P	A	P	P	P
4.30.14	7	upstream	P	A	P	P	A
	8	downstream	P	A	P	P	P
	9	reference	P	A	P	P	A
5.8.14	10	upstream	P	A	P	P	A
	11	downstream	P	A	P	P	A
	12	reference	P	A	P	P	A
5.13.14	13	upstream	P	A	P	P	A
	14	downstream	P	A	P	P	A
	15	reference	P	A	P	P	A
5.16.14	16	upstream	P	P	P	P	P
	17	downstream	P	P	P	P	P
	18	reference	P	A	P	P	P
5.21.14	19	upstream	P	A	P	P	A
	20	downstream	P	A	P	P	A
	21	reference	P	A	P	P	A
5.27.14	22	upstream	P	A	P	P	A
	23	downstream	P	A	P	P	P
	24	reference	P	A	P	P	A
6.3.14	26	downstream	P	A	P	P	P
	27	reference	P	A	P	P	A
6.10.14	29	downstream	P	A	P	P	P
	30	reference	P	A	P	P	P
6.17.14	32	downstream	P	A	P	P	A
	33	reference	P	A	P	P	P
6.24.14	35	downstream	P	A	P	P	P
	36	reference	P	A	P	P	A
7.1.14	38	downstream	P	A	P	P	P
	39	reference	P	A	P	P	A

P Presence of marker A Absence of marker

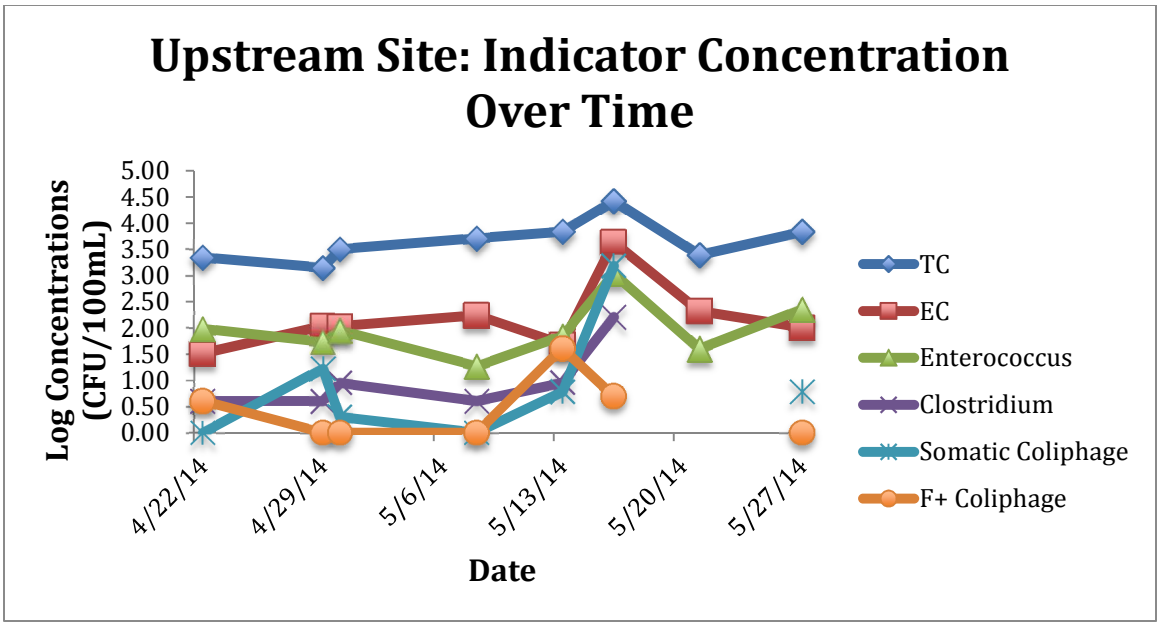

Figure 9. Indicator concentrations over time for all water samples collected at site upstream of creek B flow.

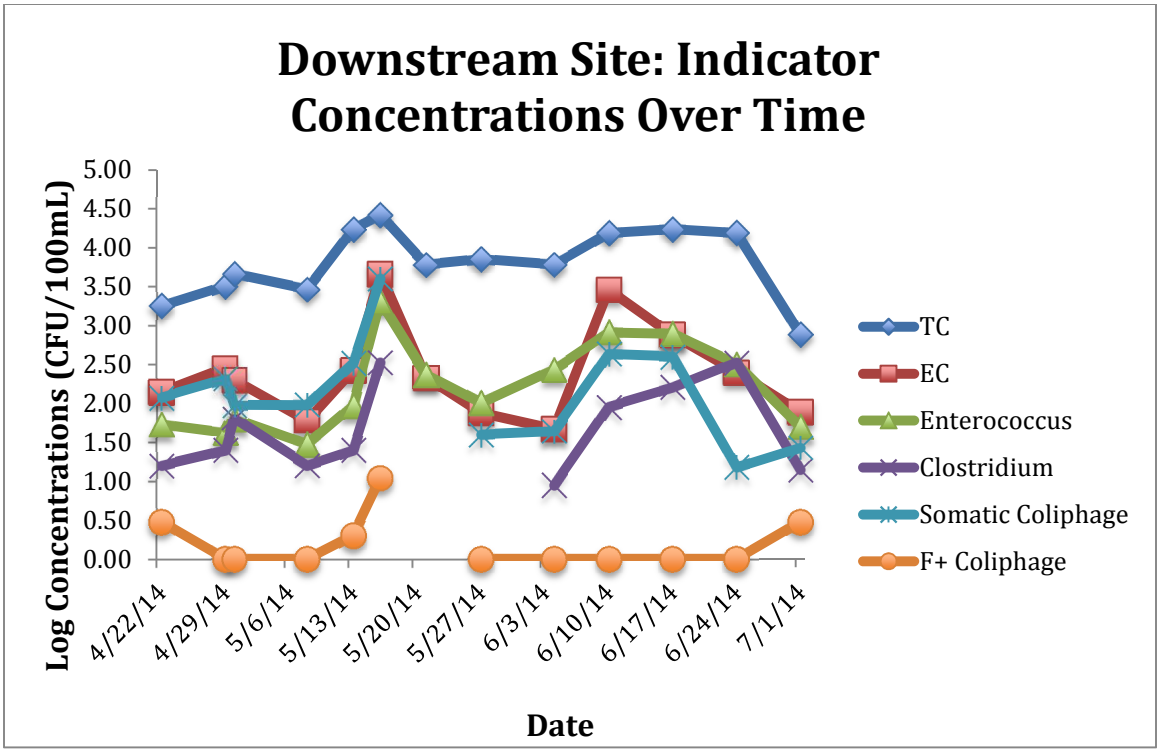

Figure 10. Indicator concentrations over time for all water samples collected at site downstream of creek B flow.

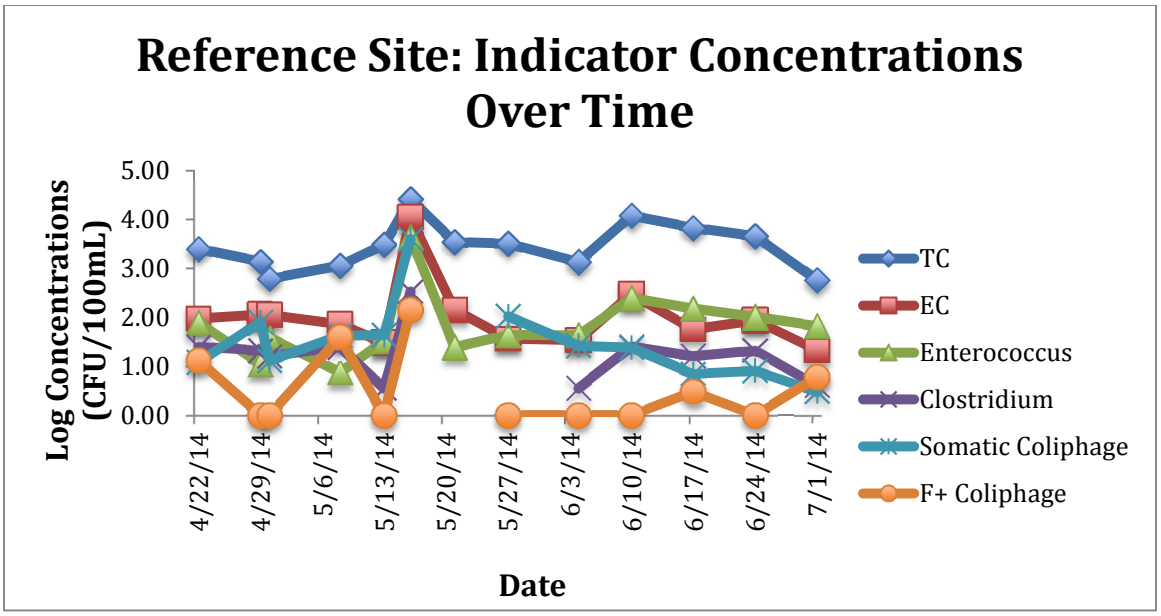

Figure 11. Indicator concentrations over time for all water samples collected at site reference along creek A flow.

Table 19.

Frequency and Percent of Tested Samples Out of Compliance with North Carolina Regulatory Thresholds across Microorganisms

Location and # of samples collected	E. coli >235 CFU/100mL* N(%)	Enterococci >61 CFU/100mL* N(%)
Upstream (N=8)	1 (13%)	5 (63%)
Downstream (N=13)	6 (46%)	9 (70%)
Reference (N=13)	2 (15%)	6 (46%)

*Standard Threshold Value criteria for freshwater
*Bacteria concentrations were quantified by an MPN method and these MPN concentrations are considered euivalent to CFU concentrtaions, hence the usage of the retatined EPA terminology of CFU/100mL

Table 20.

Differences between Sites Comparing Indicators That Have the Same Conclusions or Different Conclusions When Looking at Different Indicators for Violation of State Standards

Upstream Site		*E. coli*	
		No Violation	Violation
Enterococcus	No Violation	3	0
	Violation	4	1
Downstream Site		*E. coli*	
		No Violation	Violation
Enterococcus	No Violation	3	1
	Violation	4	5
Reference Site		*E. coli*	
		No Violation	Violation
Enterococcus	No Violation	7	0
	Violation	4	2

Same Conclusion

Different Conclusion

Table 21.

Means and Standard Deviations for Fecal Indicator Bacteria Analyzed between Three Creek Sites

	Enterococcus (MPN/100mL)	**Total Coliforms (MPN/100mL)**	***E. coli* (MPN/100mL)**	**F+ coliphages (PFU/100mL)**	**Somatic coliphages (PFU/100mL)**	**Clostridium (MPN/100mL)**
Creek Site	Mean (SD) Sample #	Mean (SD) Sample #	Mean (SD) Sample #	Mean (SD) Sample #	Mean (SD) Sample #	Mean (SD) Sample #
Upstream	1.97(0.53) n=8	3.65(0.40) n=8	2.19(0.64) n=8	0.36(0.58) n=8	0.84(1.03)* n=8	0.98(0.62) n=6
Down-stream	2.21(0.58) n=13	3.81(0.45) n=13	2.41(0.61) n=13	0.18(0.32) n=13	2.11(0.64)* n=13	1.67(0.57) n=11
Reference	1.82(0.69) n=13	3.45(0.48) n=13	2.02(0.69) n=13	0.54(0.72) n=13	1.52(0.77) n=13	1.23(0.56) n=11
	P=0.146	*P*=0.322	*P*=0.124	*P*=0.273	*P*=0.005	*P*=0.283

P-value based on ANOVA test

*Differences based on Tukey-Kramer test ($p<0.05$)

Discussion

MST Markers in the Water Environment

Our results demonstrate that we could detect the biosolid microbial markers in surface waters near biosolid land application sites. Additionally, the surface waters tested were positive for traditional indicators, but we could not assess the impact of land application due to our inability to link pollution to its source. Despite detection, the biosolid microbial markers may not be specific to biosolids materials, particularly under field conditions where there are other sources of pollution; therefore, we cannot conclude association with biosolids.

Some of the suggested markers were found at the reference sites and there is no evidence that the indicator concentrations were different between sites. Each site had at least one indicator microorganism that was out of compliance of North Carolina standards (Table 19). The downstream sample site had the most samples out of compliance and was most frequently out of compliance (Table 19). For this data, it was not useful to interpret frequencies. However, because we had so few samples, even with a non-significant result there is still some evidence to support the need for further studies with more samples.

Based on our findings, the marker targeting an uncultured *Archaea* (OTU016) appears most promising as an indicator of biosolids, although not as an exclusive source because digested animal wastes may also be sources. Specifically, marker OTU016 was absent in untreated animal manure (previous chapter) but present in biosolid samples (previous chapter) and digested animal wastes. Additionally, OTU016 appeared in only 2 of the 34 samples collected from upstream and

downstream sites (samples 16 and 17, respectively), both collected following a heavy rainfall (2.24in). It is not clear if the upstream site could have been influenced by run-off during the storm event so it is difficult to interpret the positive sample. In comparison, the sample (sample 18) collected on the same day at the reference creek (reportedly receiving no biosolids) was negative for OTU016. Based on our findings, an association to biosolids run-off cannot be determined and additional samples, including samples that capture rain events, are needed to assess the utility of OTU016 as a biosolid MST marker.

Contrary to the presence of OTU016 in only samples 16 and 17, the other candidate markers (OTU001, OTU009, and OTU040) were consistently found in the upstream, downstream, and reference samples. Again, if these markers are associated with biosolids materials then one would not expect to find the presence of these markers in the upstream site, which should be above biosolid land-applied fields. Similarly, we would not expect to find the presence of markers associated with biosolids at the reference site because this site reportedly is not near land-applied fields. We may not have captured true upstream and downstream locations relative to the application site and the reference site may not be free of biosolids. Additionally, the other markers (OTU001, OTU009, and OTU040) that were present at all of the sites could be present in soils, which we did not analyze. We cannot conclude an association with biosolid materials based on our findings.

One of the limitations of this study is our knowledge about the topography of the field site and surface water hydrology information about the creeks sampled, information that would have been useful as we interpret the presence/absence of the

biosolid microbial markers in water samples and its association to biosolid materials. Additionally, we were not able to collect any soil samples to do a comprehensive analysis of potential sources of the candidate biosolid microbial markers, which could possibly be present in soil. Biosolids at the land-applied sites also were not sampled, therefore the presence of the biomarkers in this potential biosolids source was not confirmed. Hence, soil and biosolids (at land-applied sites) data are needed to better assess and interpret the results. This study was an exploratory study to determine if biosolid microbial markers could be detected in the surface water environment, which we were able to demonstrate. A larger-scale study (including sampling the biosolids source and soils and capturing true rain events) is necessary to conclude an association with biosolids material before these markers could be used as MST markers. Included in this larger scale study should also be collection of groundwater samples because we did not have access/resources to collect this data—this should be considered as a means of exposure and should be collected in a future study.

An additional finding was the presence of HF183 in surface water samples collected from selected sites. HF183 was also present in biosolid samples from a previous study (previous chapter). The presence of HF183 in biosolids and its presence in surface waters suggest this may potentially be a marker for the presence of biosolids on fields that animals graze because it can distinguish human vs. animal sources. The sporadic presence of HF183 in various water samples, regardless of location, could possibly be explained due to confounding factors such as leaking septic tanks that could contribute to human fecal pollution. For future

studies, a sanitary survey is essential to account for all fecal pollution sources that contribute to water quality. An example of this is outlined in the World Health Organization's Water Safety and Sanitation Safety plans, which provides a holistic approach to determine the impact of fecal waste sources on the environment and possible risks of exposure for the public.[83]

Prior to MST technologies, researchers had to rely on traditional fecal indicators to determine environmental water quality but these methods are not able to distinguish sources of pollution. Previous field research investigated the impact of land application of biosolids on groundwater using traditional indicators such as *E. coli* and Enterococcus.[45,47,80,81] In contrast, our study found evidence of non-traditional markers of biosolids that may be more specific to digested waste. The present study preliminarily describes a combination of OTU016 and HF183 as a potential source-tracking tool capable of distinguishing biosolids among sources of pollution in surface water. Combining the HF183 MST marker with a biosolid-sensitive marker could be a first step in determining microbial occurrence and following microbial transport after land application. A small number of studies on bioaerosols have successfully linked novel markers to biosolid piles[8,42,50] and one study applied markers in a field study using MST techniques to track microorganisms during high wind events.[51] The latter supported the feasibility of using MST indicators unique to biosolids as aerosol source tracking markers for tracking off-site migration of biosolids following land application. Additionally, the USGS conducted a study investigating the impact that land application of biosolids has on a watershed in North Carolina and cited the need for methods to distinguish

sources of fecal pollution.[77] The surface waters analyzed by USGS were high in quantity for the traditional indicators tested, so the authors could not assess the impact of land application without the ability to link pollution to its source.[77] The report concluded the need to have markers capable of distinguishing biosolid materials from other sources of pollution, thereby supporting the benefits of this research.

Sensitivity and specificity are important parameters to consider in developing novel markers for MST. Sensitivity is defined as the ability to detect the presence of a targeted source when the source is present, whereas specificity is the ability to not detect the targeted source when the source is not present. A sensitivity/specificity analysis was conducted in Chapter 3. However, a larger scale study is necessary to provide conclusive results on feasibility of the potential biosolid microbial markers. This larger study should include testing of digested animal wastes because these samples appear to cross-react with the proposed markers in our preliminary analysis. Previous studies have not identified a perfectly (100%) sensitive and specific marker[67] and the candidate biosolids markers used here are also not 100% specific—they appear to cross-react in other treated waste samples such as digested and composted animal wastes. However, the combination of a marker sensitive to digested waste products (OTU016) and a marker specific to human wastes (HF183) may provide a tool that is sensitive and specific enough to detect and track biosolids in aquatic environments.

Strengths and Limitations

Though novel, these methods and results may not be generalizable due to differences in geography, topography, hydrology, treatment processes at different wastewater treatment plants, land application practices, and water sources. In an attempt to address some of these limitations we sampled various types of biosolids, with a range of treatments that represented the most common management practices. Meteorological factors were an additional challenge to consider in conducting this field study. Because hot and dry summer conditions caused stagnant creek water as well as creek water evaporation, we had to discontinue sampling after July 1, 2014, resulting in a small analytic sample size.

Nonetheless, the present research is innovative and expands the body of literature on MST techniques. To the best of our knowledge no study has used MST to detect and track novel biosolid markers in surface waters following land application. We applied high-throughput sequencing techniques to identify microorganisms that are typically not investigated and we were successful in demonstrating detection of these markers in surface waters. Our approach may eventually have the potential to provide tools to wastewater treatment plants and the USEPA to inform best biosolids management practices.

Future Research

The presence of biosolid marker OTU016 in biosolids and the presence of it in water samples following a rain event could suggest off-site migration of uncultured *Archaea* and possibly other microbial markers following land application. We did not measure the DNA from pathogenic bacteria; therefore, testing alongside pathogen

measures to see how well they correlate with our suggested biosolid markers would be needed to help assess potential health risks associated with land application. Survivability under different environmental conditions (including testing land-applied biosolids sources, soil and more rain event samples) and spatial and temporal scales (for various regions) are also suggested as next steps for future research.

A larger-scale study assessing the effectiveness of combining markers (e.g., OTU016 and HF183) as potential biosolid occurrence and source tracking markers is needed that detect and quantify occurrence and follow transport of microorganisms in the environment after land application. Additionally, thorough land surveillance including a sanitary survey would help in the assessment of potential sources of pollution and, consequently, a well-informed evaluation of the best microbial detection of occurrence and source-tracking markers. Future studies that consist of a larger library of diverse samples could also help in designing a marker that is host-sensitive and host-specific. Because MST is an area of research that is growing, it requires additional investigation to properly assess the impact that biosolid land application has on the environment and the various routes of transport. If subsequent research can prove the feasibility of these or other biosolid markers, then the resulting information can advise practices to enhance water quality.

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Wright for their assistance in collecting and analyzing the water samples; and Billy Gerhard for his assistance with the HF183 assays.

CHAPTER 5. CONCLUSION

Summary of Findings

The overall aim of our research was to develop novel biosolid microbial markers to investigate occurrence of these markers in environmental surface waters after land application. We identified potential microbial markers that were biosolid-sensitive based on their presence in biosolids. However, the potential markers were not biosolid-specific given their cross-reactivity with treated animal waste. Therefore, we could not validate a MST marker for biosolids material. One candidate marker currently designated as OUT016 appears that it could be useful when no other treated (digested, composted) wastes are on site or when used in combination with a human-specific marker such as HF183. Source tracking is a useful tool capable of distinguishing specific sources of pollution. To our knowledge, however, no study has used MST biomarkers to detect and track biosolids in surface waters following land application. This is of particular interest and suggests that microbial detection and source tracking in water is a key area of research that needs to be further explored. Therefore, to better understand modes of microbial occurrence in the environment after land application of biosolids and its potential impact on public health, our study findings may inform further investigation of water as a route of microbial transmission by developing new microbial indicators specific to biosolids and using microbial source tracking of such biomarkers as a method for detection.

Findings from this research require further investigation into the use of a sensitive and specific biosolids indicator that will also aid in tracing the contaminant to its source of pollution because current traditional indicators may not be as effective at such tracing or evaluating potential risk to the public. Additionally, the current traditional fecal indicators used for water quality assessment are not biosolid source–specific and the development of novel biosolid-specific indicators could contribute to science and provide a useful tool for the USEPA and wastewater treatment plants to better regulate and monitor the land application of biosolids.

Potential Benefits

Albeit this research is at an early stage in development and evaluation of feasibility of potential biosolid markers, our findings fill a critical knowledge gap by providing evidence on the potential exposure to biosolids materials and environmental impacts associated with the land application of biosolids. This research investigated evidence of microbial contamination of ambient waters providing data that could lead to a larger scale exposure assessment study. The WWTPs selected are dedicated to safe disposal and committed to creating effective ways of communicating to the public in addressing community concerns pertaining to biosolids. Hence, this line of research could benefit WWTPs by providing information on current biosolids practices and impact, potentially offering useful tools for in-house monitoring and contributing to scientific evidence that could be useful in addressing community concerns.

Current studies in NC are investigating the impact on surface and groundwater due to biosolids land application. [77] These studies are using traditional

fecal indicators that are not biosolids-specific; therefore, this exploratory research suggests an approach (MST) to better detect and track potential off site migration of biosolids material.

Innovation

Microbial source tracking (MST) methods are still under development; however, these techniques are useful and necessary in identifying sources of pollution. MST methods are especially important in cases where water quality is in question with a potential risk of exposure to the public from fecal contamination. Therefore, using MST markers to detect occurrence track sources of microorganisms following biosolids application to their ultimate fate would be transformative in addressing concerns associated with disposal of biosolids.

To the best of our knowledge this research is the first attempt, using our approach, to detect the occurrence of biosolid microbial markers in the water environment after land application to surface waters using MST techniques. In doing so, novel indicators were developed that were sensitive but not necessarily specific to biosolids material, a first step in distinguishing biosolids waste from other wastes in the environment. Albeit preliminary, the combined usage of a potential anaerobic digested biosolid marker from OTU016 and the human-specific marker, HF183, is innovative and eventually, this line of research could contribute to the overall understanding of the impact land application of biosolids has on the environment. Additionally, if the feasibility of biosolid microbial markers is proven (as useful MST markers), this approach could provide useful tools for wastewater treatment plants to better regulate and monitor the land application of biosolids.

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